IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Tapas Mukhopadhyay, *et al.*	Group Art Unit: 1642
Serial No.: 10/043,877	Examiner: B. J. Fetterolf
Filed: January 9, 2002	Atty. Dkt. No.: INRP:095US
For: ANTIHELMINTHIC DRUGS AS A TREATMENT FOR HYPERPROLIFERATIVE DISEASES	

THIRD DECLARATION OF TAPAS MUKHOPADHYAY, SUNIL CHADA, ABNER MHASHILKAR, AND JACK A. ROTH UNDER 37 C.F.R. §1.131

We, Tapas Mukhopadhyay, Sunil Chada, Abner Mhashilkar, and Jack A. Roth, hereby declare as follows:

1. We are the joint inventors of the subject matter claimed in the above-referenced patent application, U.S.S.N. 10/043,887, filed January 9, 2002.

2. We previously submitted a declaration to set forth facts demonstrating that we both conceived the idea of the invention as reflected in the claims of the above-referenced patent application and determined that it functioned, prior to March 9, 1999.

3. In the present declaration we are submitting facts demonstrating that we steadily progressed in our research to confirm that our invention functioned in an animal model, which was in accordance with our initial understanding. We continually and

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diligently conducted these studies from the time we conceived of our invention until the time our U.S. Provisional patent application, U.S.S.N. 60/261,346, was filed on January 11, 2001. Evidence of our diligence is set forth in Exhibits 1-3, discussed in detail below.

4. Submitted as Exhibit 1 to this declaration is a copy of a draft manuscript of our experiments and results, entitled "Potent Induction of Apoptosis by Anthelmintics in Human Lung Cancer Cells: Involvement of Wild-Type p53 and p21 Kinase Inhibitor." The studies set forth in this manuscript and the preparation of this manuscript took place prior to January 14, 2000.

5. Submitted as Exhibit 2 to this declaration is a copy a series of experiments and results involving the use of benzimidazoles in the treatment of p53 wild type expressing tumor cells, ending with animal models, as evidenced by the laboratory notebook of Dr. Jiichiro Sasaki, who worked under the direction of Dr. Tapas Mukhopadhyay. These experiments took place between November 10, 2000 and September 9, 2001.

6. Submitted as Exhibit 3 to this declaration is a copy of a draft manuscript of our experiments and results, including in animal models, entitled "Mebendazole: A Novel Microtubule Agent Having Potent Antitumor Activity," which was submitted for publication on October 25, 2001.

7. Exhibit 2 shows the preparation and results of a series of experiments pertaining to the benzimidazole drug, mebendazole (labeled MZ) for the treatment of cancer. Experiments pertaining to the treatment of cancer cells with mebendazole are listed on the following dates: November 11, 2000; November 15, 2000; December 5, 2000; December 8, 2000; December 12, 2000; January 11, 2001; January 12, 2001; January 18, 2001; February 10, 2001; February 20, 2001; February 23, 2001; February 26, 2001; March 2-6, 2001; March 19, 2001; March 20, 2001; March 29-31, 2001; April 3-4, 2001, April 14, 2001; April 19, 2001; April 26, 2001; June 6, 2001, June 7, 2001; June 16, 2001; July 24, 2001; July 26-28, 2001; August 1, 2001; August 4, 2001; August 5, 2001; August 7, 2001; August 31, 2001; August 31, 2001 and September 9, 2001. Of note, the last two entries pertain to the use of mebendazole in animal models.

8. Exhibit 3 shows the draft manuscript pertaining to the treatment of cancer cells with mebendazole on the inhibition of human tumor xenografts in mice. See Exhibit 3, Abstract, page 2. In accordance with our in vitro data, our animal model data showed that Mebendazole inhibited lung cancer growth. For example oral administration of mebendazole to mice previously injected with A549 lung cancer cells resulted in an 80% reduction in tumor count as compared to the control. See Exhibit 3, Results, page 10-11.

9. All work disclosed in the invention disclosure form was conducted in the United States of America.

10. Therefore, the invention as reflected in claims 75-77, 83-106, 161-162 and 184 of the above-referenced patent application was conceived of prior to March 9, 1999 and diligently reduced to practice.

11. We hereby declare that all statements made by our own knowledge are true and all statements made on information and belief are believed to be true and further that statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment under § 100 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

Date	Tapas Mukhopadhyay
Date	Sunil Chada
Date	Abner Mhashilkar
Date	Jack A. Roth

EXHIBIT 1

Potent induction of apoptosis by anthelmintics in human lung cancer cells: involvement of wild-type p53 and p21 kinase inhibitor[1]

Tapas Mukhopadhyay[2] and Jack A. Roth

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Running Title: Bendimidazole-induced apoptosis

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The abbreviations used are: MZ, (methyl 5-benzoylbenzimidazole-2-carbamate; FZ, methyl 5-(phenylthio)-2-benzimidazole carbamate (fenbendazole); TUNEL, terminal-deoxynucleotidyltransferase (TnT)- mediated dUTP-biotin nick end labeling; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; Hoechst 33342, benzidine; NSCLC, non-small cell lung cancer; DMSO, dimethylsulfoxide; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; MOPS, morpholinepropanesulfonic acid; PI, propidium iodide.

Summary

We have studied the effect of the broad-spectrum anthelmintic benzimidazoles on the regulation of apoptosis in the human lung cancer cell lines. In this study, the in vitro effect of the benzimidazole compounds fenbendazole and mebendazole, on human lung cancer cell lines was determined. These drugs dramatically inhibited the growth of lung cancer cells in culture. Western blot analysis done using specific antibodies against Bcl-2, Bcl-xl, Bax, RB, cdc2, Cdk2, cyclin A, cyclin D, and p53 showed that treatment with fenbendazole and mebendazole did not alter the levels of any of these proteins except p53. The drug treatment also induced a dose- and time-dependent nuclear accumulation of wild-type p53 whose kinetics correlated well with the induction of apoptotic cell death. The effect of these benzimidazoles was further assessed in a number of human cell lines. Interestingly, only cell lines containing the wild-type p53 were highly sensitive to growth inhibition and apoptosis after benzimidazole treatment. The presence of wild-type p53 correlated well with enhanced growth arrest, micronucleation, and p53-dependent apoptosis in drug-treated cells. In addition, p53, MDM2 and $p21^{Cip1/WAF1}$ protein levels significantly increased by 24h after drug treatment. However, cell lines carrying mutated p53 were quite resistant to the cytotoxic effect of the drugs. Restoration of wild-type p53 function made tumor cells more sensitive to FZ and MZ induced apoptosis. The ability of benzimidazole to induce apoptosis in HeLa and SiHa cell lines, which express HPV-E6 protein as a dominant negative factor for p53-mediated cell death, was diminished. Thus, our collective findings strongly suggest that a p53-dependent mechanism contributes to the cytotoxicity induced by benzimidazoles in human cancer cells.

Benzimidazoles are broad-spectrum anthelmintics that display excellent activity against parasitic nematodes and, to a lesser extent, cestodes and trematodes (1). Bendimidazoles are effective antiprotozoal agents and have antifungal activity (2). It is currently believed that benzimidazoles exert their cytotoxic effects by binding to the microtubule system and disrupting its functions (3)(4). The suggestion that tubulin is a target for benzimidazoles has been supported by the results of drug-binding studies using enriched extracts of helminth and mammalian tubulin (3). Moreover, competitive drug-binding studies using mammalian tubulin have shown that benzimidazoles compete for colchicine binding and inhibit the growth of L1210 tumor cells in vitro (5)(3). However, benzimidazoles are selectively toxic to nematodes when administered as anthelmintics but are not toxic to the host (1). In contrast, benzimidazoles suppress the in vitro polymerization of mammalian tubulin (2). Differences in the affinity between host and parasite macromolecules for benzimidazole (6)(7) as well as the pharmacokinetics of benzimidazoles between the host and the parasite have been suggested as the factors responsible for the selective toxicity of benzimidazoles (8) but the actual molecular basis of this selective toxicity remains unclear.

Of all the proteins whose loss of function is associated with cancer development, p53 is the best known. In its wild-type form, it may function as a critical regulator of genotoxic stress and apoptosis (9)(10). Studies of the wild-type protein have shown that DNA damage or oxidative stress can increase the cellular accumulation of this protein by increasing its stability in stressed cells (10)(11)(12). Increases in the level of the p53 protein may in turn directly facilitate DNA repair, or they may indirectly inhibit cell-cycle progression or induce apoptosis (10)(12). Conversely, loss of p53 function may allow damaged cells to survive and permit DNA damage to accumulate, further promoting cellular transformation (11)(13)(14)(15). Therefore, genotoxic stress surveillance and concomitant p53 accumulation are important primary processes in damaged cells. In the study described here, we exposed a number of human lung cancer cell

lines of differing p53 status to benzimidazoles to evaluate the cytotoxicity of the drugs and found that the frequency of apoptosis was greater in the benzimidazoles-exposed cells. We also correlated the involvement of the p53 gene with the degree of drug sensitization and found that cell lines containing a functional p53 gene were more sensitive to benzimidazoles.

Benzimidazoles drugs are commercially available and show remarkable safety when used as anthelmintics in the treatment of many veterinary and human helminthiases. It is therefore possible that these drugs could be used clinically to inhibit the growth cancer cells. Our results demonstrate that BZs rapidly induces the production of the wild-type p53 protein in human lung cancer cell lines and concomitantly induces apoptosis.

Experimental Procedures

Materials-Methyl-5-benzoylbenzimidazole-2-carbamate (mebendazole [MZ]) and methyl-5-(phenylthio)-2-benzimidazolecarbamate (fenbendazole [FZ]) were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were purchased from Sigma unless otherwise indicated.

Cell Culture-Non-small cell lung cancer (NSCLC) cell lines were used in our studies. All except A549 were gifts from Drs. Adi Gazdar and John Minna (The University of Texas Southwestern Medical Center, Dallas, TX). All other cell lines were obtained from the American Type Culture Collection (Rockville, MD). In all cases, cell lines were grown according to the directions provided by suppliers. All culture media were supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.) and antibiotics (100 mg/ml of streptomycin and 100 IU/ml of penicillin BRL). Mebendazole and fenbendazole were dissolved in dimethylsulfoxide (DMSO) and then diluted in phosphate-buffered saline (PBS) (1:1). When reagents containing DMSO were used, an equal volume of DMSO was added to the control cells.

In vitro cell culture and proliferation assay-All cell lines were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum and 5% CO^2. All experiments were done when the cells were 70% confluent. For cell growth measurements, $5x10^4$ cells were plated in each well of six-well plates. Control cells and cells treated with benzimidazoles (0.05 μg/ml) were trypsinized and counted using a hemocytometer. Experiments were done in triplicate, and the mean and standard deviation were determined by standard methods. Using the Curve Fit 1.3 program, the 50% growth inhibitory concentrations (IC_{50}) were extrapolated from a plot of the percent of control cell growth (triplicate determinations) versus drug concentration after 24 h of treatment.

RNA Isolation and Northern Blot Analysis-Total RNA was isolated from subconfluent cultures using the guanidinium thiocyanate method (3). After this, 20 μg of the RNA was electrophoresed in a denaturing 1.2% agarose/morpholinepropanesulfonic acid (MOPS)-

formaldehyde gel, transferred onto a nitrocellulose membrane, and hybridized to ^{32}P-radiolabeled p53 cDNA probes, as described elsewhere (16). The ^{32}P-labeled probes were generated using random primers (>8 x 10^8 cpm/μg). Blots were washed at 65°C in 2x standard saline citrate (SSC) for 30 min and then washed twice at 60°C in 0.1% sodium dodecyl sulfate (SDS) and 0.1X SSC. The cDNA probes used were 1.2-kb human p53 cDNA and an 800-bp fragment of human p21 cDNA.

Antibodies-The following antibodies were used: mouse anti-p21 monoclonal antibody WAF-1(Ab-1) Oncogene Sciences (Cambridge, MA); mouse monoclonal anti-Cyclin A (Sigma St Louis, MO), rabbit antihuman cyclin D (Upstate Biotech. Inc.) mouse anti-RB monoclonal antibody (Pharmingen , San Diego, CA), and mouse anti-c-myc monoclonal antibody (Invitrogen, Carlsbad, CA). Mouse anti-BCL-2 (100) monoclonal antibody, rabbit anti-Bcl-xl (S-19) polyclonal antibody, rabbit anti-Bax (N-20) polyclonal antibody, mouse anti-MDM2 (SM P14) monoclonal antibody, mouse anti-2 (100) monoclonal antibody, mouse anti-p53 (Bp53-12) monoclonal antibody, mouse anti-Cdk-2 (M2-G) goat polyclonal, antibody, and mouse anti-Cdc-2 p34 monoclonal antibody were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Amersham International (Arlington Heights, IL).

Nuclear Staining Assay- Cells were seeded onto chamber slides and treated with various reagents, after which cell monolayers were washed twice with ice-cold PBS (pH 7.4). Thereafter, cells were fixed for 5 min at 20°C in 10% formalin. The PBS washing step was then repeated once. To stain the nuclei, the cells were incubated for 10 min with 10 μg/ml of Hoechst 33342 and then washed with PBS. Coverslips seeded with the stained cells were mounted in 80% glycerol in PBS containing 1 mg/ml P-phenylenediamine and examined with a Nikon epifluorescence microscope.

Apoptotic assay and *TdT FACS analysis*-Apoptotic assay was done using M30 CytoDEATH

apoptotic cell death assay kit (Boehringer Mannheim). Cells were grown on chamber slides, control and benzimidazole treated cells were stained with Mouse monoclonal antibody(clone M30) as per manufacturer's instructions. Apoptotic cells were examined with a Nikon microscope and photomicrographed. For TdT FACS analysis control and treated cells were collected by trypsinization, washed in PBS, and fixed overnight in 70% ethanol. The next day, cells were rehydrated in PBS for 30 min, centrifuged, and resuspended in PBS. For DNA analysis, propidium iodide (PI) was added at 50 μg/ml, and the cells were incubated in the presence of RNase A (15 mg/ml for 30 min at 37°C). To detect DNA strand breaks associated with apoptosis, cells were fixed in 1% formaldehyde for 15 min at 4°C, rinsed in PBS, and stored at 4°C in ice-cold 70% ethanol. Before staining, the cells were washed in PBS, and 10^6 cells were resuspended in 50 ml of cacodylated buffer containing 100 μ/ml TdT enzyme and 0.5 nM biotin-16 dUTP for 30 min at 37°C. Cells were washed in PBS and resuspended in 100 ml of 4X SSC containing 2.5 mg/ml fluoresceinated avidin, 0.1% Triton X-100, and 5% dry fat milk and then incubated at room temperature for 30 min in the dark. Finally, cells were washed in PBS and resuspended in PI buffer. Flow cytometry was carried out in a fluorescence-activated cell sorter (Epics Elite; Coulter, Inc., Hialeah, FL).

Immunohistochemical Staining-Cells were seeded onto glass coverslips and fixed as described above. The cells were blocked at 37°C for 30 min with 2% bovine serum albumin, 5% fetal bovine serum, and 5% normal goat serum in PBS. The cells were then incubated at room temperature for 45 min with anti-p53 (Ab-2) antibody (1:1000 dilution) in blocking buffer and washed with PBS. The cells were then incubated with HRP-conjugated goat anti-mouse IgG secondary antibody (Amersham). After washing, the immunocomplex was detected by an avidin-biotin complex kit, and slides were mounted as described above.

Cell Lysates and Immunoblotting-Cells were grown in 6-cm dishes, cultured, and treated as described above. To prepare the whole-cell lysates, the medium was removed. Then, cells were

washed twice with ice-cold Tris-buffered saline (TBS) (150 mM NaCl, 10 mM Tris; pH 7.6) and lysed with 0.5 ml of lysis buffer (10 mM Tris, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM benzamidine, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 1 mM sodium orthovanadate) for 15 min. The lysed cells were then transferred to 1.5-ml microtubes and centrifuged at 15,000 × g for 10 min at 4°C. Then, the supernatants were collected, mixed with Laemmli's sample buffer, and subjected to western blot analysis as described elsewhere (5). Blots were probed with anti-p53 monoclonal antibody B p53-12 (Santa Cruz Biotechnology, Inc.), and immunocomplexes were detected using an Enhanced Chemiluminescence kit (Amersham) according to the manufacturer's directions. Blots were then reprobed, this time with anti-actin monoclonal antibody (Amersham), to show that protein loading was equal. After that, blots were again reprobed, this time with anti-WAF monoclonal antibody (Pharmingen, San Diego, CA).

Pulse-Chase Experiments-Cells were treated with 0.05 μg/ml fenbendazole or mebendazole for 24 h. After this, control (mock-treated) and drug-treated H460 cells were incubated with 10 μg/ml cycloheximide in drug-free medium for different times and then processed to obtain total cell extracts. Finally, samples were denatured by boiling them in SDS-loading buffer (100 mM Tris, pH 6.8; 2% SDS; 0.1% bromphenol blue; 10% glycerol; 25 μM β-mercaptoethanol) and loaded onto a 10% SDS-polyacrylamide gel. Blots were probed with anti-p53 monoclonal antibody B p53-12 (Santa Cruz Biotechnology, Inc.) and anti-actin monoclonal antibody (Amersham). The immunocomplexes were detected using the Enhanced Chemiluminescence kit according to the manufacturer's directions as described above. The intensities of the bands were quantitated with a PhospholmagerTM using ImageQuantTM Software (Molecular Dynamics, Sunnyvale, CA).

Results

BZ-induced Apoptosis in Human NSCLC Cell Line H460-We tested the cytotoxic effect of fenbendazole and mebendazole on the human NSCLC cell line H460. After treatment with fenbendazole (0.05 μg/ml) or mebendazole (0.05 μg/ml) for 48 h, the cells became rounded and loosely attached to the plates, suggesting the cells were viability. Further analyses of the cell samples showed that the cells were undergoing apoptosis. Most of the morphological hallmarks associated with apoptosis were detectable, including cell shrinkage, DNA fragmentation, and chromatin condensation (Fig. 1A). The DNA strand breaks typical of the apoptosis, were also demonstrated by TUNEL and benzimidine staining (Fig. 1, A and B). The morphological changes produced by FZ and MZ treatments were indistinguishable. Gross morphological changes associated with a loss of viability were observed by in 24h after treatment, whereas signs of apoptosis, detected by DNA staining and TUNEL assays, became apparent by 24 h after drug treatment. Because of the close structural resemblance between FZ and MZ (i.e.,both have the benzamidazole core structure), it is not surprising that they both induced apoptosis and the nuclear accumulation of p53 (Fig. 1C).

Induction of Nuclear Accumulation of p53-Both fenbendazole and mebendazole showed an apoptotic effect on the H460 cells. To rule out the possibility that fenbendazole and mebendazole were affecting other proteins known to play a role in various apoptotic pathways, we evaluated the effect of these drugs on a panel of such proteins. Many of these proteins are known activators or suppressors (e.g., Bcl-2, Bax, RB) of apoptosis and have already been shown to be expressed in these cells. Specifically, the levels of these proteins were evaluated by western blot analyses following fenbendazole and mebendazole treatments. Although the levels of nuclear p53, p21, and MDM2 were enhanced, there were, interestingly, no changes in the levels of Bcl-2, cyclin A, cyclin D, Cdc 2, Bcl-xl, Bax, RB, or Cdk-2 (Fig. 2). The protein level of the phosphorylated form of RB also remained unchanged after treatment for 24 h (Fig. 2).

Because fenbendazole and mebendazole specifically increased the amount of nuclear p53 detectable in the 460 cell line (Fig. 3), the apoptotic effect of these drugs on these NSCLC cells was presumably p53 dependent. There was also a positive correlation between the ability of fenbendazole and mebendazole to induce apoptosis and the ability of these agents to mediate the nuclear accumulation of p53. As a result of p53 protein accumulation, the p53-regulated genes were also expressed at much higher levels (Fig. 3). MDM2 and p21 levels analyzed by western blot analysis after fenbendazole treatment increased.

Kinetics of Induction of p53 and Apoptosis-The effect of fenbendazole and mebendazole on the nuclear accumulation of p53 appeared to be gradual and was not significant within the first hour of exposure. However, it became significantly detectable at about 16 h and peaked at 24 h (Fig. 4A). The appearance of p53 in response to drug treatment also coincided with the initiation of apoptosis, which was detectable by 24 h. Northern blot analysis, however, revealed no significant changes in the p53 mRNA levels (Fig. 4B), indicating that the nuclear accumulation of p53 was not due to an increase in p53 transcripts. As evidence that the p53 induced in these cells was functional, the level of the transcript for one of the p53 target genes, p21/WAF1, was significantly increased at the time the p53 level peaked (Fig. 4B).

The induction of p53 became detectable at a dose of 0.01 μg/ml fenbendazole (Fig. 5A). However, the induction appeared to occur abruptly, which suggests the presence of a cooperative mechanism. Similar observations were made under serum-free conditions, suggesting that the source of the cooperative effect was not factors in the serum (data not shown). As predicted in light of the northern blot experiment, the steady-state p21 level (in parallel with the p53 protein level) did increase significantly over that in the control experiment in the H460 cells but not in the mutant p53-carrying H322 cells. The results of the DNA fragmentation assay, which assessed the effect of fenbendazole in inducing of apoptosis, and the assays of nuclear accumulation of p53 correlated well (Fig. 5B).

Increased Half-life of p53 in Drug-treated Cells-Because fenbendazole and mebendazole did not seem to affect the transcriptional rate of the p53 gene, we evaluated the effect of these agents on the stability of p53 in H460 cells and noted that both agents were able to prolong the half-life of the p53 protein in H460 cells. Because the results were similar for fenbendazole and mebendazole treatments, only data obtained from fenbendazole-treated cells are presented. Specifically, the half-life of the wild-type p53 in untreated H460 cells was about 6-8 h compared to 24 h in the drug-treated cells (Fig. 6). The former finding is consistent with the previously reported half-life of p53 in a number of cancer cell lines, which far outstripped the 20-to 30-min half life seen in normal fibroblasts (17).

Selective Induction of Apoptosis by Fenbendazole and Mebendazole in Tumor Cells Carrying Wild-type p53-Studies to determine the IC_{50} for fenbendazole and mebendazole were performed on a panel of six different human cancer cell lines: two carrying the wild-type p53 gene and actively expressing the p53 protein, (H460 and A549); one marked by homozymes deletion of the p53 gene and lacking p53 gene expression (H358); two expressing mutant p53 (H322 and H596); and one in which wild-type p53 is inactivated by human papilloma virus E6 protein. Comparison of the concentrations of bendimidazoles necessary to inhibit the growth of the different cell lines by 50% (IC_{50}) indicated that wild-type p53 containing cell lines were 2-to 7-fold more sensitive than the p53-mutated or deleted cell lines (Table I). A dose of 166 nM (~ 0.05 mg/ml) was chosen for these studies because it had been shown previously that this concentration was sufficient to induce wild-type p53 after 24 h of treatment.

To further investigate the relationship between the induction of functional p53 and the subsequent apoptosis mediated by these drugs, we posited that if BZs require wild-type p53 in order to exert their effect, then fenbendazole and mebendazole would not be cytotoxic to the many tumor lines producing a mutated p53 protein. To investigate this possibility, human cell lines derived from NSCLC origins of differing p53 status were further analyzed by a 5-day cell

growth assay. Results for three cell lines (H460, H322, H1299) are shown in Fig. 7. H322 is a human lung adenocarcinoma line that produces a mutant p53 protein. The mutant p53 protein is generally more stable (18)(19) and this was reflected in the present case by the presence of higher amounts of the p53 protein in the nucleus of H322 cells prior to drug treatment (Fig. 7). H1299 is a p53 gene-deleted human NSCLC cell line and does not express any p53 protein. However, fenbendazole and mebendazole induced nuclear accumulation of p53 only in the H460 cell line, which carries wild-type p53 genes. Fenbendazole and mebendazole therefore appeared to be significantly effective in killing wild-type p53-containing cancer cells.

An analysis of 18 human tumor cell lines (Table II) was performed, which showed that these drugs had an effect on nuclear accumulation of p53 only in the cell lines carrying the wild-type p53 gene. Both drugs induced some degree of growth inhibition; rather than apoptosis, in cell lines that contained mutated or deleted p53; however, they induced greater growth inhibition in cell lines containing wild-type p53: As expected, HeLa and SiHa cervical cancer cell lines containing wild-type p53 along with HPV-E6 protein were less sensitive to the inductive effect of these drugs on apoptosis and p53 accumulation.

In order to further confirm that bendamidazoles works through p53 mediated pathway the synergistic effect of Ad5p53 and Fenbendazole on tumor cell growth was examined in four human lung cancer cell lines that differed in p53 status but were all transduced with Ad5p53: H1299 (*p53* deleted), H322 (*p53* mutated), H460 (wt *p53*) and A549 (wt *p53*). Because our initial dose-response studies indicated that 0.05 ug/ml FZ induced high levels of wt *p53* protein expression in H460 cells without toxicity, we used this concentration for all our proliferation assays. In those assays, growing, cultured cells were trypsinized and plated (10^4 cells/well) and then infected the next day with Ad5p53 at 1 MOI. Viral supernatant was then added, after which cells were incubated for 24 h, washed with PBS, fed fresh medium or incubated with medium containing FZ for 24 h, washed again, and fed fresh medium. In contrast, uninfected cells

(controls) were treated with FZ for 24 h, washed with PBS, fed fresh medium, and then subjected to a 3-day growth assay (Fig.8A).

Our experimental results indicated that d1312 (empty vector) had no effect in combination with FZ (Fig. 8B). When the four lung cancer cell lines were transiently infected with 1 MOI Ad5p53 for 24 h, no growth suppression was observed, regardless of *p53* status. When all four lines were treated with FZ alone for 24 h, the *p53*-mutated and deleted cells were not growth inhibited, whereas the wt p53 H460 cells was significantly so. However, a striking growth inhibition was observed in all four cell lines when the Ad5p53-transduced cells (1 MOI, 24 h) were treated with FZ for 24 h and were grown in normal medium for a 3-days growth assay. Our findings suggest that transducing of Adp53 will induce efficient p53-mediated killing of tumor cells in the presence of p53 superinduction by FZ. The A549 cells (containing wt p53) showed 11% apoptotic cells following FZ treatment. Low dose wild-type Adp53 had no apoptotic effect, whereas the A549 cells showed 30% apoptotic cells death 48 h after combination treatment, as shown by terminal deoxynucleotidal transferase (TDT) staining analysis via fluorescence-activated cell sorting (FACS) as shown in Fig. 8C. These results suggest that FZ works though a p53 dependent pathway.

Inhibition of apoptotic effect by the dominant negative factor E6-To provide more direct evidence that the cytotoxic effect of fenbendazole and mebendazole correlates with the availability of functional p53 in the cell, we studied HeLa and SiHa ovarian cancer cells line that produce the E6 protein, which acts as a dominant negative factor for wild-type p53. This production of E6 is attributable, at least in part, to a dominant negative mechanism involving the degradation of the endogenous wild-type molecule. In this experiment, the HeLa (HPV-18) and SiHa (HPV-16) cells were treated with fenbendazole for 48 h, and the total cell proteins were subjected to immunoblot analysis for p53 protein production. However, no induction of p53 production was observed **(Fig. 9)**, suggesting that the E6-mediated degradation of p53 was

utilizing a path different from that used in normal cells. Because these results could also have been due to a decrease in the MDM2 protein level after fenbendazole treatment, we reprobed the membrane with MDM2 monoclonal antibody. However, MDM2 protein production remained low in both control and fenbendazole-treated HeLa and SiHa cells, whereas MDM2 production in the H460 cells increased several times with the increase in the p53 protein levels. Further, in these dominant negative lines, the level of the p21 protein did not increase above the base level after treatment with these drugs. This clearly showed that fenbendazole-mediated cell killing had become less effective in cells producing the wild-type p53-inactivating E6 protein, thereby limiting the ability of fenbendazole and mebendazole to mediate apoptosis. This finding may, in part, explain why killed those cells containing wild-type p53. However, one cannot rule out the possibility that a pathway other than the p53 pathway is also involved in the cytotoxic effect of these drugs.

Discussion

We observed that the BZs fenbendazole and mebendazole selectively induced apoptosis in human NSCLC cells. Moreover, several lines of evidence in our study suggested that this apoptotic effect was mediated, at least in part, by the p53 protein. First, the kinetics of the induction of apoptosis and p53 accumulation were similar. Second, these compounds failed to induce apoptosis in mutant p53 cell lines and were also ineffective in inducing p53 production. Third, the production of an E6 protein acting as a dominant negative factor in the production of the wild-type p53 protein was sufficient to counteract the cytotoxic effect of these drugs. Finally restoration of wild-type p53 function by an adenoviral vector made tumor cells more sensitive to FZ and MZ induced apoptosis.

It is widely believed that restoring or enhancing wild-type p53 functions in tumor cells may one day be used to successfully treat many human cancers (20). Certainly, the *p53* gene is the most commonly mutated gene in human cancer (21)(22), and the resultant mutation of the p53 protein often inactivates tumor suppressor function, even though 40-50% of tumors may still retain copies of the wild-type *p53* gene. Recent evidence further suggests that, despite the production of functional wild-type p53 protein in human cancer cells, the amounts of wild-type p53 protein produced are so low that the protein, appears unable to execute its normal apoptotic function. In an effort to activate its apoptotic functions, it would therefore be of interest to determine the effect of fenbendazole and mebendazole on the trafficking of p53 protein between the cytoplasmic and nuclear compartment in these cells. Because phosphorylation of Ser 15 has been implicated as a mechanism underlying the increased stability of the p53 protein, the effect of these anthelmintics on the phosphorylation pattern of p53 is currently being investigated in our laboratory.

It is already known that DNA-damaging agents such as etoposide and Adriamycin induce production of the p53 protein in cell lines harboring the wild-type gene. These agents are

thought to enhance the level of p53 in cells by increasing its stability (23). This increase in stability has been borne out by immunofluorescence studies in fenbendazole-treated cells, which showed that there was a strong increase in p53 staining in the nucleus. Interestingly, our experiments also showed that the anthelmintic drugs we studied increased the half-life of p53 in H460 cells from 6 to 24 h.

The kinetics of p53 induction in H460 cells by these drugs and by MG132, a well-known proteosome inhibitor, are similar (data not shown). This strongly suggests that BZs inactivate a selective degradation pathway, thereby triggering the inductive effect on p53 in these cells. Therefore, it remains a possibility that the molecular target for BZs is not a kinase but a point in the p53-proteosome pathway of protein degradation where they can interfere with its progression.

This is the first report demonstrating that anthelmintics regulate the apoptotic function of wild-type p53 in human NSCLC cells. The fact that structurally related BZ analogues share this property of inducing apoptotic activities suggests that there is a unique and specific structural determinant of apoptosis embedded in their chemical structure and that this effect is mediated through wild-type p53. It has been suggested that the role of p53 in cell-cycle arrest may be distinct from its role in apoptosis and that each of these functions may be served by discrete domains in the molecule (24)(25). For example, a mutation in the p53 protein that inactivates its function in apoptosis may not necessarily affect its function in cell-cycle arrest. Interestingly, however, we observed in the present study that the induction of p53 protein in the drug-treated cells paralleled the induction of G1 cell-cycle arrest and apoptosis. It has been reported that Bax is a target gene for p53 (26). However, unlike p21, in the NSCLC wild-type p53 cell lines we studied here, the level of Bax protein, remained unchanged and was not upregulated by p53. Nevertheless, this result is consistent with the finding in some cell types that p53 does not appear to increase Bax levels (27).

The BZs are a group of structurally similar compounds that have been shown to possess antimitotic activity in vitro and in vivo (28) but also to induce a presumably p53-independent mechanism. In addressing this contradiction, we found no changes in the steady-state level of Bcl-2, RB, and Cdk-2 in H460 cells upon treatment with BZs for 24 h. However, it is possible that BZs regulate some of these proteins in a cell-type-dependent manner. In fact, the list of proteins that seem to play important roles in various apoptotic pathways is growing rapidly. However, whether BZs can regulate apoptotic genes other than those we have studied remains to be investigated.

In our study, fenbendazole and mebendazole induced p53 accumulation but failed to induce apoptosis in E6-expressing (HeLa and SiHa) cells. This inability may be attributed to the specific ubiquitinization of E6-AP and the subsequent degradation of wild-type p53, actions that perhaps protect these cells from the effects of BZs. Alternatively, this inability may suggest the presence of an additional downstream step in the apoptotic pathway. To distinguish between these two possibilities, more human cancer cell lines carrying the wild-type p53 genes need to be evaluated.

Acknowledgments

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References

1. Bossche, V.H., Thienpont, P.G., and Janssens, P.G. (1985) *Chemotherapy of Gastrointestinal Helminths*, Berlin

2. Davidse, L.C. (1986) *Annu.Rev.Phytopathol.* **24**, 43-65

3. Lacey, E. (1988) *Int.J.Parasitol.* **18**, 885-936

4. Friedman, P.A. and Platzer, E.G. (1980) *Biochim.Biophys.Acta* **630**, 271-278

5. Nare, B., Liu, Z., Prichard, R.K., and Georges, E. (1994) *Biochem.Pharmacol.* **48**, 2215-2222

6. Russell, G.J., Gill, J.H., and Lacey, E. (1992) *Biochem.Pharmacol.* **43**, 1095-1100

7. Kohler, P. and Bachmann, R. (1981) *Mol.Biochem.Parasitol.* **4**, 325-336

8. Gottschall, D.W., Theodorides, V.J., and Wang, R. (1990) *Parasitol.Today* **6**, 115-124

9. Liu, Z.G., Baskaran, R., Lea-Chou, E.T., Wood, L.D., Chen, Y.W., Karin, M., and Wang, J.Y. (1996) *Nature* **384**, 273-276

10. Yonish-Rouach, E. (1996) *Experientia.* **52**, 1001-1007

11. Hainaut, P. (1995) *Curr.Opin.Oncol.* **7**, 76-82

12. Bellamy, C.O., Malcomson, R.D., Harrison, D.J., and Wyllie, A.H. (1995) *Semin.Cancer Biol.* **6**, 3-16

13. Gottlieb, T.M. and Oren, M. (1996) *Biochim.Biophys.Acta.* **1287**, 77-102

14. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C.C. (1991) *Science* **253**, 49-53

15. Griffiths, S.D., Clarke, A.R., Healy, L.E., Ross, G., Ford, A.M., Hooper, M.L., Wyllie, A.H., and Greaves, M. (1997) *Oncogene* **14**, 523-531

16. Lubega, G.W. and Prichard, R.K. (1990) *Mol.Biochem.Parasitol.* **38**, 221-232

17. Freedman, D.A. and Levine, A.J. (1998) *Mol.Cell Biol.* **18**, 7288-7293

18. Hinds, P.W., Finlay, C.A., Quartin, R.S., Baker, S.J., Fearon, E.R., Vogelstein, B., and Levine, A.J. (1990) *Cell Growth Differ.* **1**, 571-580

19. Kraiss, S., Spiess, S., Reihsaus, E., and Montenarh, M. (1991) *Exp.Cell Res.* **192**, 157-164

20. Lowe, S.W. (1995) *Curr.Opin.Oncol.* **7**, 547-553

21. Nigro, J.M., Baker, S.J., Preisinger, A.C., Jessup, J.M., Hostetter, R., Cleary, K., Bigner, S.H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F.S. , Weston, A., Modali, R., Harris, C.C., and Vogelstein, B. (1989) *Nature* **342**, 705-708

22. Takahashi, T., Nau, M.M., Chiba, I., Birrer, M.J., Rosenberg, R.K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A.F., and Minna, J.D. (1989) *Science* **246**, 491-494

23. Fritsche, M., Haessler, C., and Brandner, G. (1993) *Oncogene* **8**, 307-318

24. Haupt, Y., Rowan, S., Shaulian, E., Vousden, K.H., and Oren, M. (1995) *Genes Dev.* **9**, 2170-2183

25. Wagner, A.J., Kokontis, J.M., and Hay, N. (1994) *Genes Dev.* **8**, 2817-2830

26. Miyashita, T. and Reed, J.C. (1995) *Cell* **80**, 293-299

27. Canman, C.E., Gilmer, T.M., Coutts, S.B., and Kastan, M.D. (1995) *Genes Dev.* **9**, 600-611

28. Lacey, E. and Watson, T.R. (1985) *Biochem.Pharmacol.* **34**, 3603-3605

29. Negrini, M., Sabbioni, S., Haldar, S., Possati, L., Castagnoli, A., Corallini, A., Barbanti-Brodano, G., and Croce, C.M. (1994) *Cancer Res.* **54**, 1818-1824

30. Mitsudomi, T., Steinberg, S.M., Nau, M.M., Carbone, D., D'Amico, D., Bodner, S., Oie, H.K., Linnoila, R.I., Mulshine, J.L., Minna, J.D., and Gazdar, A.F. (1992) *Oncogene* **7**, 171-180

31. Perdomo, J.A., Naomoto, Y., Haisa, M., Fujiwara, T., Hamada, M., Yasuoka, Y., and Tanaka, N. (1998) *J.Cancer Res.Clin.Oncol.* **124**, 10-18

32. Chandar, N., Billig, B., McMaster, J., and Novak, J. (1992) *Br.J.Cancer* **65**, 208-214

33. Bressac, B., Galvin, K.M., Liang, T.J., Isselbacher, K.J., Wands, J.R., and Ozturk, M. (1990) *Proc.Natl.Acad.Sci.U.S.A.* **87**, 1973-1977

34. Bartek, J., Iggo, R., Gannon, J., and Lane, D.P. (1990) *Oncogene* **5** , 893-899

35. Wolf, J.K., Mills, B.G., Bast, R.C., Jr., Roth, J.A., and Gershenson, D.M. (1998) in *Ovarian Cancer* (Sharp, F., Blackett, T., Berek, J., and Bast, R., eds) pp. 259-271, Isis Medical Media Ltd., Oxford

36. Hamada, K., Sakaue, M., Alemany, R., Zhang, W.W., Horio, Y., Roth, J.A. , and Mitchell, M.F. (1999) *Gynecol.Oncol.* **63**, 219-227

37. Stratton, M.R., Moss, S., Warren, W., Patterson, H., Clark, J., Fisher, C., Fletcher, C.D.M., Ball, A., Thomas, M., Gusterson, B.A., and Cooper, C.S. (1990) *Oncogene* **5**, 1297-1301

38. Tarunina, M. and Jenkins, J.R. (1993) *Oncogene* **8**, 3165-3173

Figure Legends

Fig. 1. Induction of apoptosis. H460 cells were treated with 0.05 mg/ml mebendazole or fenbendazole and their morphological changes associated with apoptosis identified. *A*, widespread loss of viability was noted by gross examination of the cells at 24 h after treatment (upper panel). The cells were photographed using a phase-contrast light microscope. Chromatin condensation was noted under a fluorescence microscope at

24 h after treatment the condensation was shown by staining the nuclei with Hoechst 33342 fluorescent dye) (middle panel). DNA strand breaks were detected at 24 h after treatment using a direct immunoperoxidase method (lower panel). *B*, induction of apoptosis was assessed by TdT FACS analysis in the human lung cancer cell line H460. Cells were harvested after 24 h of exposure to fenbendazole and mebendazole (0.05 mg/ml) and the apoptotic cells quantitated after TdT staining as described in Experimental Procedures.

Fig. 2. Effect of drug treatment on the level of various proteins. Western blot analysis of various proteins in H460 cells before and after exposure to 0.05 μg/ml fenbendazole or mebendazole for 24 h. Whole-cell extracts were used for the analysis.

Fig. 3. Ability of fenbendazole and mebendazole to induce the expression of p53 protein and its target genes. *A*, H460 cells were treated with fenbendazole or mebendazole for 24 h (0.05 μg/ml), and the proteins from both control and treated cells were analyzed for p53 protein and p53 target gene expression. Increased p53 expression correlated with enhanced p21 and MDM2 protein levels. *B*, chemical structures of mebendazole and fenbendazole.

Fig. 4. Fenbendazole-and mebendazole-induced expression of p53 protein and mRNA. *A*, cell extracts were prepared from H460 cells exposed to fenbendazole (0.05 μg/ml) for various times (1 to 24 h). p53 protein was detected by immunoblot analysis. The position of the p53 protein is indicated by an arrow. *B*, northern blot analysis of p53 and p21 mRNA levels in H460 cells treated with 0.05 μg/ml fenbendazole or mebendazole after 24 h of treatment. Total RNA was extracted and used (20 μg per lane) for the

analysis.

Fig. 5. **The effect of fenbendazole in inducing apoptosis.** *A*, immunoblot analysis of p53 protein in the H460 human lung cancer cell line. The proteins were extracted from cells treated with different concentrations of fenbendazole and mebendazole for 24 h. ■ - represent FZ treated ; ▲- represent control untreated cells. *B*, NSCLC cell lines of differing p53 status were treated with 0.05 μg/ml fenbendazole for 24 h and then stained with p53 monoclonal antibody. The nuclear accumulation of wild-type p53 was associated with nuclear fragmentation that resulted in apoptosis.(A, C, and E) Untreated control cells and fenbendazole Z-treated cells (B, D, and F); H460 (wild-type p53)--upper panel; H322 (mutated p53)--middle panel; H1299 (deleted p53)--lower panel

Fig. 6. **Effect of fenbendazole and mebendazole on stability of the p53 protein.** *A*, stability of the p53 protein in H460 cells upon fenbendazole and mebendazole treatment. H460 cells were treated with 0.05 μg/ml fenbendazole for 24 h, and then both untreated control and treated cells were washed with PBS and treated with 25 μg/ml cycloheximide. After that, cells were harvested at different times. The total cell protein was extracted and analyzed on a 10% SDS-polyacrylamide gel, followed by western blot analysis using p53 and actin monoclonal antibodies (actin served as an internal control). The experiment was repeated twice, producing similar results both times. *B*, p53 protein stability in cells. Data from the pulse-chase experiments were quantitated with a PhosphoImager using ImageQuantTM software. Circles and triangles represent data from fenbendazole-treated and control samples, respectively.

Fig. 7. **Effect of fenbendazole and mebendazole the induction of cell growth and nuclear accumulation of wild-type p53 protein.** ***A,*** The NSCLC cell lines H460 (wild-type p53), H322 (mutant p53), and H1299 (deleted p53) were exposed to fenbendazole (filled bar) or mebendazole (hatched bar) at concentrations of 0.05 μg/ml. The cells were plated onto six-well plates and their viability determined by trypan blue extrusion. Cells were then counted on a hemocytometer. The values shown are the means ± standard deviation of triplicate samples. Duplicate experiments gave similar results. Using equal amounts of protein extracts taken from each cell line before and after treatment, an immunoblot analysis of nuclear p53 was also performed to determine whether these drugs induced the production of p53. The upper band corresponds to p53, and the lower band corresponds to actin protein, which was used as an internal loading control.

Fig. 8 **Synergistic effect of Ad5p53 and Fenbendazole.** Approximately 10^4 cells were seeded on tissue culture plates 24 h before drug treatment or adenoviral infection. A) Cell growth was measured in untreated controls, cells treated with 0.05ng/ml FZ and cells treated with the combination of Ad5p53 (1 MOI) and FZ . B) H322 cells were also treated with an empty adenoviral vector, dl312, in addition to Adp53 to show dl312 had no effect alone or in combination with FZ. C) A549 lung cancer cells were examined for apoptotic cell death 48 h after FZ and Adp53 treatment alone or in combination. Percent apoptotic cells were measured by TdT-FACS analysis.

Fig. 9 **Effect of E6 protein on the cytotoxicity of fenbendazole**. Production of p53, MDM2, and p21 proteins after fenbendazole treatment was examined in HeLa and SiHa cells and compared with the production of these proteins in H460 cells. The positions of the p53, MDM2, and p21 protein bands are indicated.

Table I. Sensitivity of cancer cell lines to bendimidazoles

	IC_{50}	
Phenotype and cell lines	Fenbendazole (nM)	Mebendazole (nM)
p53 positive		
H460	152	106
A549	123	130
HeLa (p53 inactivated)	853	400
p53 mutated		
H322	816	871
H596	643	601
p53 deleted		
H358	654	893

[a]Concentrations of drugs (in nanomolars) required to inhibit growth by 50% after 1 day of exposure.

Table II. Effect of fenbendazole and mebendazole on p53 induction and apoptosis in human tumor cell lines of differing p53 status[a]

Cell Line	Tumor origin	p53 status	% Cell Viability		Nuclear p53	p53 Induction
			fenbendazole[b]	mebendazole[b]		
MCF-7	Breast	Wild-type (29)C	43.88 ± 7.3	24.26 ± 8.4	No	Yes
H460	Lung	Wild-type (30)C	39.16 ± 6.4	25.23 ± 7.6	Yes	Yes
H549	Lung	Wild-type (30)C	46.43 ± 13.3	39.19 ± 10.7	Yes	Yes
H322	Lung	R248H (30)	65.92 ± 4.9	68.50 ± 3.7	Yes	No
H596	Lung	R245C (30)	86.71 ± 11.5	86.19 ± 8.0	Yes	No
H226Br	Lung	R254 (31)	80.06 ± 11.1	64.24 ± 0.2	Yes	No
H1299	Lung	Deleted (30)	89.17 ± 1.1	89.18 ± 4.3	No	No
H358	Lung	Deleted (30)	88.35 ± 17.8	73.45 ± 1.8	No	No
Saos-2	Osteosarcoma	Deleted (32)	76.48 ± 5.9	65.36 ± 10.1	No	No
Hep 3B	Liver	Deleted (33)	79.49 ± 12.5	83.24 ± 1.5	No	No
SW480	Colon	R273H (21)	70.59 ± 7.6	63.63 ± 13.3	Yes	No
MDA 231	Breast	R280K (34)	65.26 ± 5.0	66.24 ± 15.8	Yes	No
SK-OV-433	Ovarian	Wild-type (35)C	52.52 ± 13.2	28.15 ± 6.1	Yes	Yes
HeLa	Cervical	Wild-type but inactivated by E6 (34)C	93.63 ± 7.4	92.42 ± 6.27	Yes	No

SiHa	Cervical	Wild-type but inactivated by E6 (36)C	94.52 ± 7.4	91.32 ± 8.6	Yes	No
RD	Rhabdomyosarcoma	R248W (37)	78.73 ± 12.0	72.24 ± 2.8	Yes	No
HT1080	Osteosarcoma	Wild-type (38)C	54.52 ± 12.0	44.49 ± 7.7	ND[d]	ND[d]

[a]The viability of the cells was measured by trypan blue extrusion cell count assay. The 100% value was derived from measurements obtained from untreated cells. Experiments were done in triplicate. p53 protein was examined by immunoblot analysis of the nuclear extracts isolated from each cell line before and after treatment with 0.05 mg/ml fenbendazole and mebendazole for 24 h.

[b]Concentration of fenbendazole and mebendazole = 0.05 mg/ml.

[c]This cell line carries a wild-type allele.

[d]ND, not done.

EXHIBIT 2

Project No. 5

Book No. 1 TITLE MZ and apoptosis

Page No. 178

(Cell) HCT116 (P53+/+) HCT116 (P53-/-) A549

(Design) (day 0) cell preparation. 1000/well/100μl media (A549), 3000/well (HCT)

(day 1) Add 50μl of media containing MZ or DMSO

(day 3) XTT assay

(Results)

HCT116(-/-) treated with MZ or DMSO; 48 hours; XTT; 1107-1110000

DMSO(%)	0	0.0005	0.001	0.0025	0.005	0.01	0.025	0.05	0.1	0.25	0.5	
1	0.817	1.124	1.128	1.169	1.26	1.223	1.192	1.088	1.289	1.223	1.266	0.7
2	1.14	1.207	1.2	1.148	1.224	1.214	1.222	1.283	1.287	1.293	1.249	1.0
3	1.129	1.209	1.189	1.2	1.263	1.241	1.152	1.213	1.214	1.231	1.346	1.1
4	1.08	1.203	1.123	1.175	1.329	1.204	1.115	1.142	1.214	1.243	1.294	0.9
5	0.542	1.157	1.13	1.16	1.205	1.292	1.232	1.232	1.254	1.215	1.182	0.6
Average	0.9416	1.18	1.154	1.1704	1.256	1.2348	1.1826	1.1916	1.2516	1.241	1.2674	0.90
SD	0.2319	0.034	0.0333	0.0174	0.042	0.0311	0.0438	0.0688	0.0331	0.0276	0.0539	0.19
MZ(ug/ml)	0	0.005	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	1
1	0.835	0.998	0.971	1.01	1.159	1.043	0.982	0.807	0.603	0.325	0.223	0.16
2	1.111	1.129	1.101	1.149	1.095	1.185	1.147	0.844	0.628	0.357	0.268	0.18
3	1.082	1.142	1.077	1.144	1.174	1.148	1.099	0.942	0.544	0.369	0.259	0.17
4	1.028	1.143	1.179	1.218	1.222	1.217	1.172	0.857	0.586	0.346	0.239	0.16
5	0.645	1.039	1.107	1.235	1.267	1.308	1.198	0.957	0.644	0.348	0.221	0.11
Average	0.9402	1.0902	1.087	1.1512	1.183	1.1802	1.1196	0.8814	0.601	0.349	0.242	0.1626
SD	0.1762	0.0602	0.0673	0.0793	0.058	0.0867	0.0762	0.0582	0.0348	0.0145	0.0188	0.0273

Average	0	0.005	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
DMSO	0.9416	1.18	1.154	1.1704	1.256	1.2348	1.1826	1.1916	1.2516	1.241	1.2674	0.9068
MZ	0.9402	1.0902	1.087	1.1512	1.183	1.1802	1.1196	0.8814	0.601	0.349	0.242	0.1626
Ratio	0	0.005	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
DMSO	1	1.2532	1.2256	1.243	1.334	1.3114	1.2559	1.2655	1.3292	1.318	1.346	0.963
MZ	1	1.1595	1.1561	1.2244	1.259	1.2553	1.1908	0.9375	0.6392	0.3712	0.2574	0.1729

To Page No.

Witnessed & Understood by me, yoshi

Date 6/5/01

Invented by Daichiro Sasaki

Recorded by Daichiro Sasaki

Date 11/10/00

TITLE Western Blot.

Project No. 5

Book No. 1

From Page No.

Sample : H460, HCT116 P53 +/+, HCT116 P53 -/- (whole cell lysate and Cytosolic/Mitochondri

Treatment : MZ 0.05ug/ml 24hours

Lysis : 1× SDS, 2ME

Volume : f apply : 50ug for Methallothioneim, 10ug for cytochrome C.

PAGE : 12.5 %.

First antibody Metallothionein ×200
Cytochrome C ×500
) 1hour at r.t.

Second antibody Anti-mouse HRP ×2000 1hour at r.t.

Exposure 15sec & 5min

Re-probe P53 & β-actin (P53 ×2000, β-actin ×5000)

To Page No.

Witnessed & Understood by me, Yoshi

Date 6/5/01

Invented by Juchiro Sasaki

Recorded by N. D. D. D.

Date 11/15/00

Project No. C
Book No. 1 TITLE XTT

from Page No.__

To make Dose response curve after treatment MZ.

Cell : H460, A549, HCT116 -/-

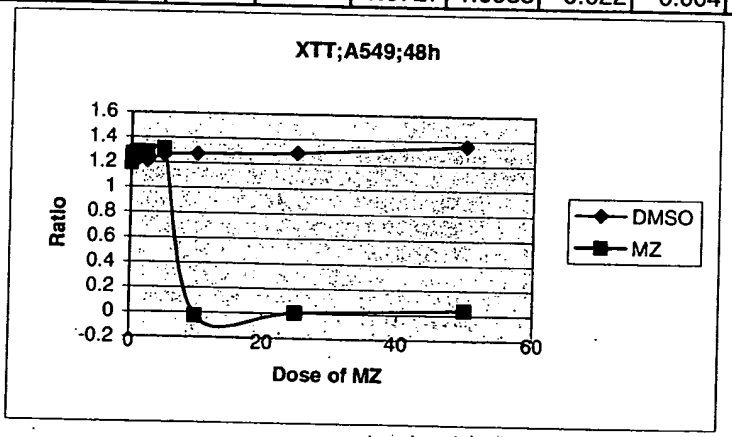
Treatment : MZ (0 – 50 μM) 48 hours exposure.

Method : XTT (2h)

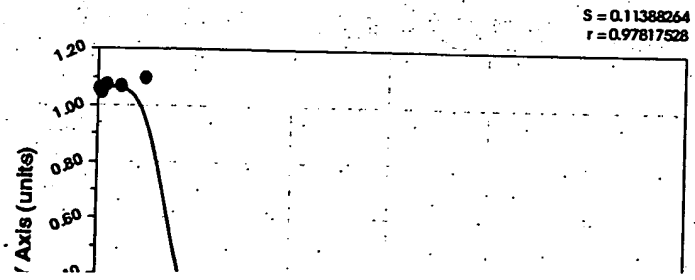
A549 treated with MZ or DMSO; 48 hours; XTT; 1201-120500

DMSO(%)	0	0.0015	0.003	0.0075	0.015	0.03	0.075	0.15	0.3	0.75	1.5
1	1.264	1.18	1.154	1.22	1.206	1.204	1.196	1.262	1.224	1.277	1.361
2	1.239	1.193	1.153	1.202	1.203	1.205	1.177	1.221	1.249	1.257	1.343
3	1.299	1.19	1.17	1.241	1.245	1.24	1.196	1.243	1.282	1.269	1.332
4	1.321	1.248	1.298	1.364	1.258	1.256	1.275	1.31	1.334	1.348	1.392
Average	1.2808	1.2028	1.1938	1.2568	1.228	1.2263	1.211	1.259	1.2723	1.2878	1.357
SD	0.0315	0.0266	0.0606	0.0634	0.024	0.0225	0.0378	0.0328	0.0412	0.0355	0.0227
MZ(uM)	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
1	1.159	1.231	1.302	1.283	1.316	1.341	1.389	1.394	-0.006	0.016	0.073
2	1.199	1.289	1.275	1.311	1.265	1.339	1.285	1.341	-0.022	0.011	0.039
3	1.195	1.202	1.205	1.231	1.204	1.21	1.24	1.26	-0.039	-0.003	0.035
4	1.22	1.205	1.193	1.238	1.209	1.235	1.206	1.247	-0.04	-0.005	0.02
Average	1.1933	1.2318	1.2438	1.2658	1.249	1.2813	1.28	1.3105	-0.027	0.0048	0.0418
SD	0.0219	0.0349	0.046	0.0329	0.046	0.0594	0.0689	0.0602	0.014	0.009	0.0194

Average	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1.2808	1.2028	1.1938	1.2568	1.228	1.2263	1.211	1.259	1.2723	1.2878	1.357
MZ	1.1933	1.2318	1.2438	1.2658	1.249	1.2813	1.28	1.3105	-0.027	0.0048	0.0418
Ratio	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1	0.9391	0.9321	0.9813	0.959	0.9574	0.9455	0.983	0.9934	1.0055	1.0595
MZ	1	1.0323	1.0423	1.0608	1.046	1.0737	1.0727	1.0983	-0.022	0.004	0.035



0	1
0.05	1.0323
0.1	1.0423
0.25	1.0608
0.5	1.0463
1	1.0737
2.5	1.0727
5	1.0983
10	-0.022



To make Dose response curve after treatment MZ -

Cell : H460, A549, HCT116 -/-

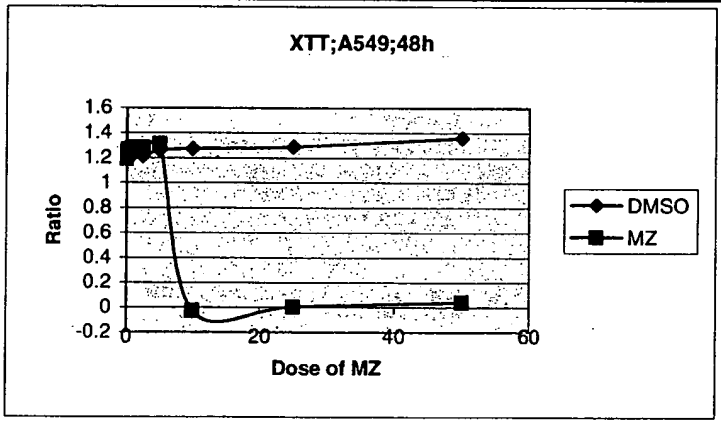
Treatment : MZ (0 – 50 μM) 48 hours exposure.

Method : XTT (2h)

A549 treated with MZ or DMSO; 48 hours; XTT; 1201-120500

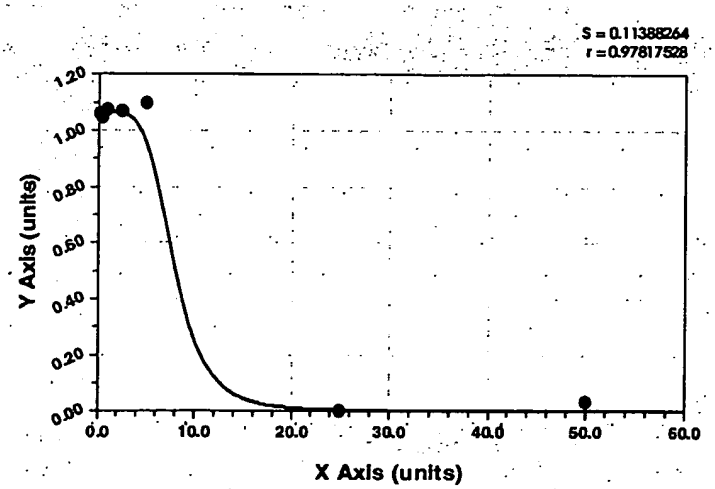
DMSO(%)	0	0.0015	0.003	0.0075	0.015	0.03	0.075	0.15	0.3	0.75	1.5
1	1.264	1.18	1.154	1.22	1.206	1.204	1.196	1.262	1.224	1.277	1.361
2	1.239	1.193	1.153	1.202	1.203	1.205	1.177	1.221	1.249	1.257	1.343
3	1.299	1.19	1.17	1.241	1.245	1.24	1.196	1.243	1.282	1.269	1.332
4	1.321	1.248	1.298	1.364	1.258	1.256	1.275	1.31	1.334	1.348	1.392
Average	1.2808	1.2028	1.1938	1.2568	1.228	1.2263	1.211	1.259	1.2723	1.2878	1.357
SD	0.0315	0.0266	0.0606	0.0634	0.024	0.0225	0.0378	0.0328	0.0412	0.0355	0.0227
MZ(uM)	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
1	1.159	1.231	1.302	1.283	1.316	1.341	1.389	1.394	-0.006	0.016	0.073
2	1.199	1.289	1.275	1.311	1.265	1.339	1.285	1.341	-0.022	0.011	0.039
3	1.195	1.202	1.205	1.231	1.204	1.21	1.24	1.26	-0.039	-0.003	0.035
4	1.22	1.205	1.193	1.238	1.209	1.235	1.206	1.247	-0.04	-0.005	0.02
Average	1.1933	1.2318	1.2438	1.2658	1.249	1.2813	1.28	1.3105	-0.027	0.0048	0.0418
SD	0.0219	0.0349	0.046	0.0329	0.046	0.0594	0.0689	0.0602	0.014	0.009	0.0194

Average	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1.2808	1.2028	1.1938	1.2568	1.228	1.2263	1.211	1.259	1.2723	1.2878	1.357
MZ	1.1933	1.2318	1.2438	1.2658	1.249	1.2813	1.28	1.3105	-0.027	0.0048	0.0418
Ratio	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1	0.9391	0.9321	0.9813	0.959	0.9574	0.9455	0.983	0.9934	1.0055	1.0595
MZ	1	1.0323	1.0423	1.0608	1.046	1.0737	1.0727	1.0983	-0.022	0.004	0.035



0	1
0.05	1.0323
0.1	1.0423
0.25	1.0608
0.5	1.0463
1	1.0737
2.5	1.0727
5	1.0983
10	-0.022
25	0.004
50	0.035

IC20	6.16
IC50	7.97
IC80	10.6



Project No. 5

Book No. 1 TITLE XTT

Page No.

To make Dose response curve after treatment MZ.

Cell : H460, A549, HCT116 -/-

Treatment : MZ (0 – 50 μM) 48 hours exposure.

Method : XTT (2h)

A549 treated with MZ or DMSO; 48 hours; XTT; 1201-120500

DMSO(%)	0	0.0015	0.003	0.0075	0.015	0.03	0.075	0.15	0.3	0.75	1.5
1	1.264	1.18	1.154	1.22	1.206	1.204	1.196	1.262	1.224	1.277	1.361
2	1.239	1.193	1.153	1.202	1.203	1.205	1.177	1.221	1.249	1.257	1.343
3	1.299	1.19	1.17	1.241	1.245	1.24	1.196	1.243	1.282	1.269	1.332
4	1.321	1.248	1.298	1.364	1.258	1.256	1.275	1.31	1.334	1.348	1.392
Average	1.2808	1.2028	1.1938	1.2568	1.228	1.2263	1.211	1.259	1.2723	1.2878	1.357
SD	0.0315	0.0266	0.0606	0.0634	0.024	0.0225	0.0378	0.0328	0.0412	0.0355	0.0227
MZ(uM)	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
1	1.159	1.231	1.302	1.283	1.316	1.341	1.389	1.394	-0.006	0.016	0.073
2	1.199	1.289	1.275	1.311	1.265	1.339	1.285	1.341	-0.022	0.011	0.039
3	1.195	1.202	1.205	1.231	1.204	1.21	1.24	1.26	-0.039	-0.003	0.035
4	1.22	1.205	1.193	1.238	1.209	1.235	1.206	1.247	-0.04	-0.005	0.02
Average	1.1933	1.2318	1.2438	1.2658	1.249	1.2813	1.28	1.3105	-0.027	0.0048	0.0418
SD	0.0219	0.0349	0.046	0.0329	0.046	0.0594	0.0689	0.0602	0.014	0.009	0.0194

Average	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1.2808	1.2028	1.1938	1.2568	1.228	1.2263	1.211	1.259	1.2723	1.2878	1.357
MZ	1.1933	1.2318	1.2438	1.2658	1.249	1.2813	1.28	1.3105	-0.027	0.0048	0.0418
Ratio	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1	0.9391	0.9321	0.9813	0.959	0.9574	0.9455	0.983	0.9934	1.0055	1.0595
MZ	1	1.0323	1.0423	1.0608	1.046	1.0737	1.0727	1.0983	-0.022	0.004	0.035

To Page No.

sed & Understood by me,	Date	Invented by	Date
yoshi	6/5/01	J.S	12/5/00
		Recorded by J.S	

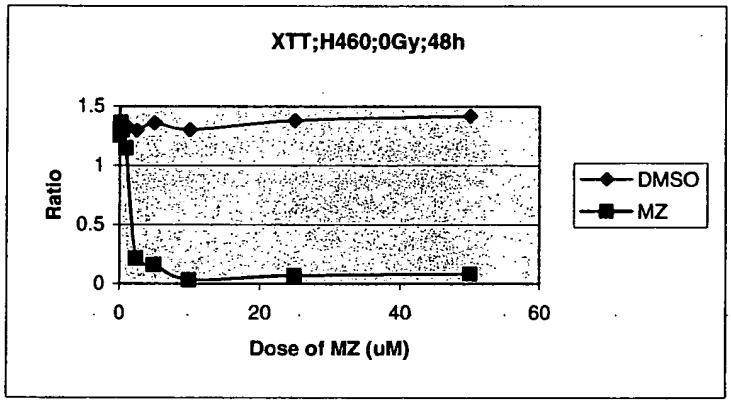
Project No. 5
Book No. 1 TITLE XTT

From Page No. To make Dose response curve and evaluate synergistic effect of M
Radiation.
Cell = H460, A549
Treatment: Radiation 0G, 5Gy, 10Gy, MZ 0-50uM, 48hours
Method = XTT (2h)

H460 treated with MZ and Radiation;Radiation:0Gy 48 hours; XTT; 1201-120500

DMSO(%)	0	0.0015	0.003	0.0075	0.015	0.03	0.075	0.15	0.3	0.75	1.5
1	1.288	1.284	1.19	1.257	1.255	1.347	1.336	1.359	1.309	1.419	1.431
2	1.228	1.265	1.244	1.251	1.254	1.274	1.25	1.312	1.271	1.344	1.381
3	1.284	1.288	1.238	1.288	1.346	1.344	1.359	1.415	1.345	1.343	1.405
4	1.273	1.283	1.371	1.341	1.293	1.352	1.241	1.349	1.279	1.406	1.455
Average	1.2683	1.28	1.2608	1.2843	1.287	1.3293	1.2965	1.3588	1.301	1.378	1.418
SD	0.0239	0.0089	0.067	0.0356	0.038	0.032	0.0517	0.0369	0.0291	0.0348	0.0277
MZ(uM)	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
1	1.207	1.243	1.277	1.342	1.34	1.049	0.172	0.165	0.036	0.072	0.106
2	1.277	1.312	1.312	1.422	1.332	1.084	0.208	0.137	0.033	0.073	0.081
3	1.256	1.302	1.351	1.366	1.258	1.104	0.247	0.175	0.03	0.064	0.077
4	1.275	1.331	1.289	1.328	1.263	1.353	0.236	0.172	0.037	0.069	0.068
Average	1.2538	1.297	1.3073	1.3645	1.298	1.1475	0.2158	0.1623	0.034	0.0695	0.083
SD	0.0282	0.0329	0.0282	0.0359	0.038	0.1203	0.029	0.015	0.0027	0.0035	0.0141

Average	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1.2683	1.28	1.2608	1.2843	1.287	1.3293	1.2965	1.3588	1.301	1.378	1.418
MZ	1.2538	1.297	1.3073	1.3645	1.298	1.1475	0.2158	0.1623	0.034	0.0695	0.083
Ratio	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1	1.0093	0.9941	1.0126	1.015	1.0481	1.0223	1.0714	1.0258	1.0865	1.1181
MZ	1	1.0345	1.0427	1.0883	1.036	0.9153	0.1721	0.1294	0.0271	0.0554	0.0662



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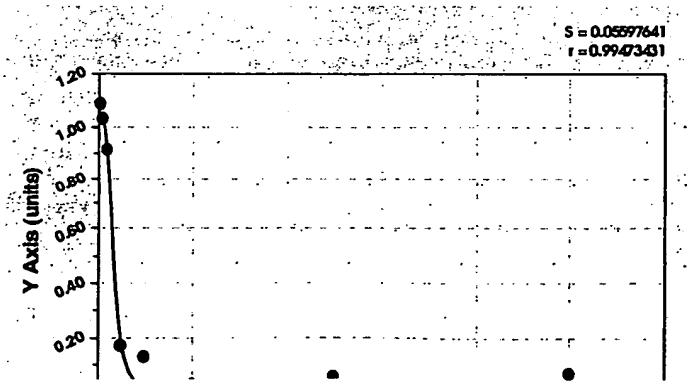
0.1	1.0427
0.25	1.0883
0.5	1.0355
1	0.9153
2.5	0.1721
5	0.1294
10	0.0271
25	0.0554
50	0.0662



Radiation

Cell : H460 , A549

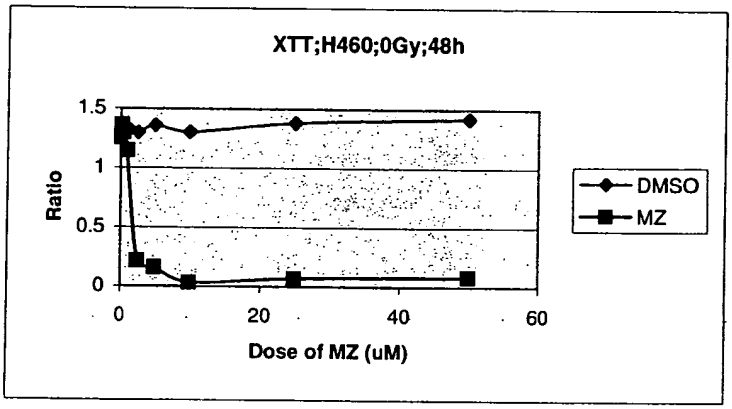
Treatment : Radiation 0G, 5Gy, 10Gy , MZ 0-50uM , 48hours

Method : XTT (2h)

H460 treated with MZ and Radiation;Radiation:0Gy 48 hours; XTT; 1201-120500

DMSO(%)	0	0.0015	0.003	0.0075	0.015	0.03	0.075	0.15	0.3	0.75	1.5
1	1.288	1.284	1.19	1.257	1.255	1.347	1.336	1.359	1.309	1.419	1.431
2	1.228	1.265	1.244	1.251	1.254	1.274	1.25	1.312	1.271	1.344	1.381
3	1.284	1.288	1.238	1.288	1.346	1.344	1.359	1.415	1.345	1.343	1.405
4	1.273	1.283	1.371	1.341	1.293	1.352	1.241	1.349	1.279	1.406	1.455
Average	1.2683	1.28	1.2608	1.2843	1.287	1.3293	1.2965	1.3588	1.301	1.378	1.418
SD	0.0239	0.0089	0.067	0.0356	0.038	0.032	0.0517	0.0369	0.0291	0.0348	0.0277
MZ(uM)	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
1	1.207	1.243	1.277	1.342	1.34	1.049	0.172	0.165	0.036	0.072	0.106
2	1.277	1.312	1.312	1.422	1.332	1.084	0.208	0.137	0.033	0.073	0.081
3	1.256	1.302	1.351	1.366	1.258	1.104	0.247	0.175	0.03	0.064	0.077
4	1.275	1.331	1.289	1.328	1.263	1.353	0.236	0.172	0.037	0.069	0.068
Average	1.2538	1.297	1.3073	1.3645	1.298	1.1475	0.2158	0.1623	0.034	0.0695	0.083
SD	0.0282	0.0329	0.0282	0.0359	0.038	0.1203	0.029	0.015	0.0027	0.0035	0.0141

Average	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1.2683	1.28	1.2608	1.2843	1.287	1.3293	1.2965	1.3588	1.301	1.378	1.418
MZ	1.2538	1.297	1.3073	1.3645	1.298	1.1475	0.2158	0.1623	0.034	0.0695	0.083
Ratio	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1	1.0093	0.9941	1.0126	1.015	1.0481	1.0223	1.0714	1.0258	1.0865	1.1181
MZ	1	1.0345	1.0427	1.0883	1.036	0.9153	0.1721	0.1294	0.0271	0.0554	0.0662



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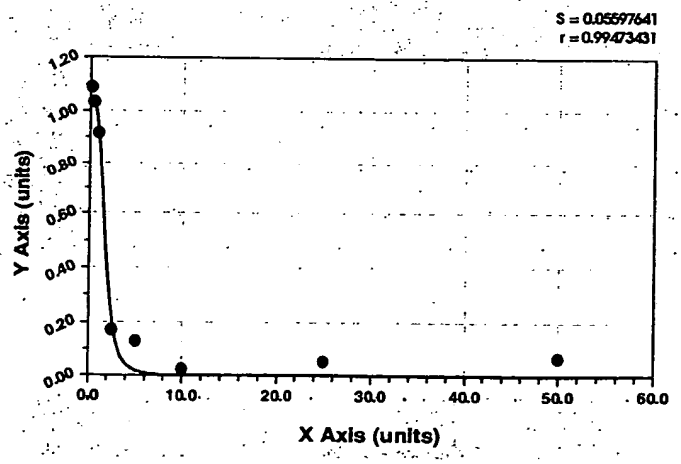
0.1	1.0427
0.25	1.0883
0.5	1.0355
1	0.9153
2.5	0.1721
5	0.1294
10	0.0271
25	0.0554
50	0.0662



IC20	1.2
IC50	1.7
IC80	2.5

om Page No. Tomako Dose response curve and evaluate synergistic effect of MZ and Radiation.

Cell : H460 , A549

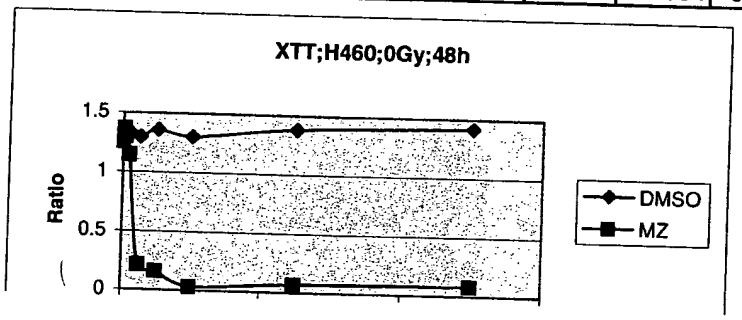
Treatment: Radiation 0G, 5Gy, 10Gy , MZ 0-50uM , 48hours

Method = XTT (2h)

H460 treated with MZ and Radiation;Radiation:0Gy 48 hours; XTT; 1201-120500

DMSO(%)	0	0.0015	0.003	0.0075	0.015	0.03	0.075	0.15	0.3	0.75	1.5
1	1.288	1.284	1.19	1.257	1.255	1.347	1.336	1.359	1.309	1.419	1.431
2	1.228	1.265	1.244	1.251	1.254	1.274	1.25	1.312	1.271	1.344	1.381
3	1.284	1.288	1.238	1.288	1.346	1.344	1.359	1.415	1.345	1.343	1.405
4	1.273	1.283	1.371	1.341	1.293	1.352	1.241	1.349	1.279	1.406	1.455
Average	1.2683	1.28	1.2608	1.2843	1.287	1.3293	1.2965	1.3588	1.301	1.378	1.418
SD	0.0239	0.0089	0.067	0.0356	0.038	0.032	0.0517	0.0369	0.0291	0.0348	0.0277
MZ(uM)	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
1	1.207	1.243	1.277	1.342	1.34	1.049	0.172	0.165	0.036	0.072	0.106
2	1.277	1.312	1.312	1.422	1.332	1.084	0.208	0.137	0.033	0.073	0.081
3	1.256	1.302	1.351	1.366	1.258	1.104	0.247	0.175	0.03	0.064	0.077
4	1.275	1.331	1.289	1.328	1.263	1.353	0.236	0.172	0.037	0.069	0.068
Average	1.2538	1.297	1.3073	1.3645	1.298	1.1475	0.2158	0.1623	0.034	0.0695	0.083
SD	0.0282	0.0329	0.0282	0.0359	0.038	0.1203	0.029	0.015	0.0027	0.0035	0.0141

Average	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1.2683	1.28	1.2608	1.2843	1.287	1.3293	1.2965	1.3588	1.301	1.378	1.418
MZ	1.2538	1.297	1.3073	1.3645	1.298	1.1475	0.2158	0.1623	0.034	0.0695	0.083
Ratio	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1	1.0093	0.9941	1.0126	1.015	1.0481	1.0223	1.0714	1.0258	1.0865	1.1181
MZ	1	1.0345	1.0427	1.0883	1.036	0.9153	0.1721	0.1294	0.0271	0.0554	0.0662



essed & Understood by me, Yoshi

Date 6/5/01

Invented by J S

Recorded by J S

Date 12/5/00

From Page No. Dose response curve after MZ Treatment and CD Treatment

All: H1299

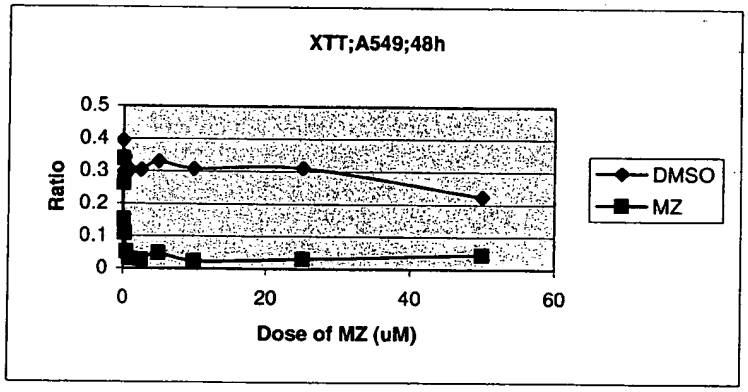
Treatment: MZ (0 – 50μM) 48hrs exposure CD (0 – 500μM)

Method: XTT

H1299 treated with MZ or DMSO; 48 hours; XTT; 1205-120800

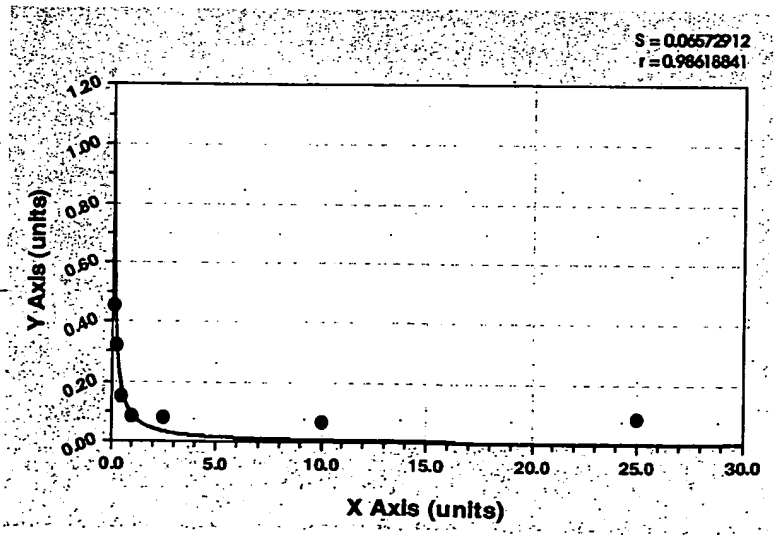
DMSO(%)	0	0.0015	0.003	0.0075	0.015	0.03	0.075	0.15	0.3	0.75	1.5
1	0.321	0.329	0.328	0.373	0.341	0.326	0.311	0.338	0.29	0.32	0.233
2	0.38	0.307	0.292	0.329	0.309	0.304	0.294	0.278	0.32	0.336	0.216
3	0.446	0.338	0.255	0.323	0.259	0.28	0.302	0.322	0.284	0.269	0.216
4	0.43	0.369	0.32	0.34	0.253	0.303	0.306	0.382	0.336	0.315	0.228
Average	0.3943	0.3358	0.2988	0.3413	0.291	0.3033	0.3033	0.33	0.3075	0.31	0.2233
SD	0.0488	0.0223	0.0286	0.0193	0.036	0.0163	0.0062	0.0372	0.0214	0.0249	0.0075
MZ(uM)	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
1	0.325	0.26	0.182	0.118	0.068	0.044	0.035	0.04	0.045	0.039	0.092
2	0.353	0.274	0.138	0.099	0.052	0.032	0.042	0.043	0.024	0.037	0.039
3	0.346	0.263	0.143	0.092	0.062	0.015	0.023	0.041	0.013	0.029	0.034
4	0.329	0.258	0.146	0.127	0.025	0.025	0.01	0.037	0.011	0.014	0.015
Average	0.3383	0.2638	0.1523	0.109	0.052	0.029	0.0275	0.0403	0.0233	0.0298	0.045
SD	0.0116	0.0062	0.0174	0.0141	0.016	0.0106	0.0122	0.0022	0.0135	0.0098	0.0286

Average	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	0.3943	0.3358	0.2988	0.3413	0.291	0.3033	0.3033	0.33	0.3075	0.31	0.2233
MZ	0.3383	0.2638	0.1523	0.109	0.052	0.029	0.0275	0.0483	0.0233	0.0298	0.045
Ratio	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1	0.8516	0.7578	0.8656	0.737	0.7692	0.7692	0.837	0.78	0.7863	0.5663
MZ	1	0.7797	0.4501	0.3222	0.153	0.0857	0.0813	0.1426	0.0687	0.088	0.133



0	1
0.05	0.7797
0.1	0.4501
0.25	0.3222
0.5	0.153
1	0.0857
2.5	0.0813
10	0.0687
25	0.088

IC20	0.03
IC50	0.11
IC80	0.4



witness

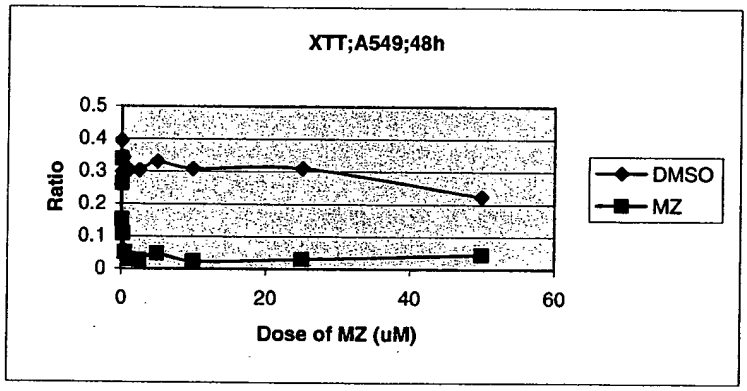
No.

om Page No. Dose respone curve after MZ Treatment and CD Treatment
All: H1299
Treatment: MZ (0 – 50 uM) 48 hours exposure CD (0 – 500 uM)
Method: XTT

H1299 treated with MZ or DMSO; 48 hours; XTT; 1205-120800

DMSO(%)	0	0.0015	0.003	0.0075	0.015	0.03	0.075	0.15	0.3	0.75	1.5
1	0.321	0.329	0.328	0.373	0.341	0.326	0.311	0.338	0.29	0.32	0.233
2	0.38	0.307	0.292	0.329	0.309	0.304	0.294	0.278	0.32	0.336	0.216
3	0.446	0.338	0.255	0.323	0.259	0.28	0.302	0.322	0.284	0.269	0.216
4	0.43	0.369	0.32	0.34	0.253	0.303	0.306	0.382	0.336	0.315	0.228
Average	0.3943	0.3358	0.2988	0.3413	0.291	0.3033	0.3033	0.33	0.3075	0.31	0.2233
SD	0.0488	0.0223	0.0286	0.0193	0.036	0.0163	0.0062	0.0372	0.0214	0.0249	0.0075
MZ(uM)	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
1	0.325	0.26	0.182	0.118	0.068	0.044	0.035	0.04	0.045	0.039	0.092
2	0.353	0.274	0.138	0.099	0.052	0.032	0.042	0.043	0.024	0.037	0.039
3	0.346	0.263	0.143	0.092	0.062	0.015	0.023	0.041	0.013	0.029	0.034
4	0.329	0.258	0.146	0.127	0.025	0.025	0.01	0.037	0.011	0.014	0.015
Average	0.3383	0.2638	0.1523	0.109	0.052	0.029	0.0275	0.0403	0.0233	0.0298	0.045
SD	0.0116	0.0062	0.0174	0.0141	0.016	0.0106	0.0122	0.0022	0.0135	0.0098	0.0286

Average	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	0.3943	0.3358	0.2988	0.3413	0.291	0.3033	0.3033	0.33	0.3075	0.31	0.2233
MZ	0.3383	0.2638	0.1523	0.109	0.052	0.029	0.0275	0.0483	0.0233	0.0298	0.045
Ratio	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1	0.8516	0.7578	0.8656	0.737	0.7692	0.7692	0.837	0.78	0.7863	0.5663
MZ	1	0.7797	0.4501	0.3222	0.153	0.0857	0.0813	0.1426	0.0687	0.088	0.133



0	1

nessed & Understood by me, Date 6/5/01 Invented by J.S Date 12/8/00
Recorded by J-S

Book No. 2 TITLE Western Blot.

From Page No.

Cell : A549

Treatment : MB 10 μM , time course (0h, 12h, 24h, 36h)

Lysate : SDS + Urea

Volume = 50 μg

PAGE : 10 %

1st Ab : caspase3 (x500)

2nd Ab : Anti-rabbit HRP (x5000) } 1h. rt.

Exposure : 5 sec and 30 sec

Re-probe : P53 & β-actin

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
Yoshi	6/5/01	J S	12/20/00
		Recorded by J S	

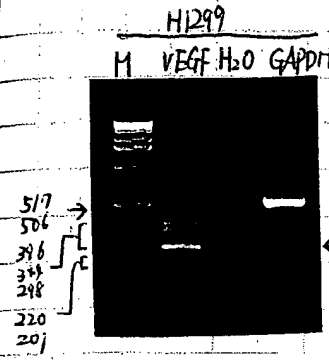
TLE RT-PCR

Project No. 5
Book No. 2

From Page No.— VEGF expression after MZ treatment

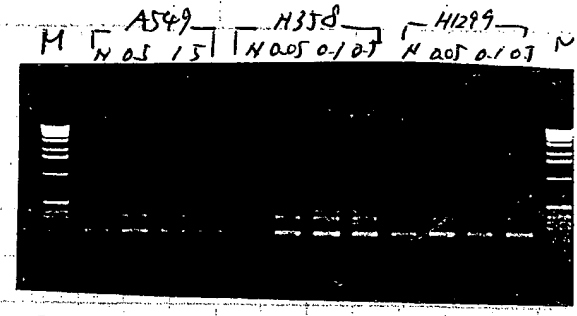
Cell: H1299, A549, H358

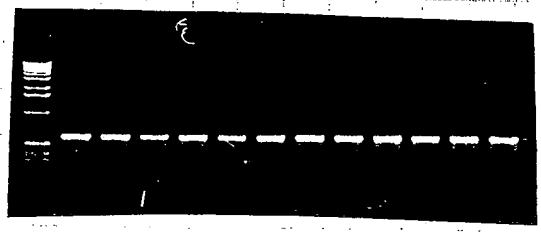
Treatment: MZ ; 24 hours



← Type I VEGF (293 bp)

(MZ: uM/ml)





BECKMAN DU-600

Date: 12/20/00
Time: 05:33

Nucleic Acid
ReadSamples Method SaveClear Print Quit

Results file: A:\WORK_RES Method name: A:\RNA100

Assay type: General Ratio and Concentration Units: ug/ml
Formula setup: VIEW Background Correction: [No]
Sampling device: None Concentration: [Yes]
Read average time: 0.50 sec Peak Pick: [No]

	Sample ID		abs 260.0 nm	abs 280.0 nm	260.0 nm / 280.0 nm	280.0 nm / 260.0 nm	Protein ug/ml	Nucleic Acid ug/ml
1	1299	N	0.3232	0.2021	1.5993	0.6253	0.0000	1292.8983
2		0.05	0.3368	0.2130	1.5809	0.6325	0.0000	1347.1895
3		0.1	0.3761	0.2364	1.5909	0.6286	0.0000	1504.3396
4		0.5	0.2643	0.1645	1.6067	0.6224	0.0000	1057.1166
5	358	N	0.4051	0.2456	1.6493	0.6063	0.0000	1620.2367
6		0.01	0.3709	0.2234	1.6603	0.6023	0.0000	1483.5201
7		0.1	0.4368	0.2643	1.6527	0.6051	0.0000	1747.2542
8		0.5	0.3481	0.2126	1.6371	0.6108	0.0000	1392.2246

A549

Sample ID		abs 260.0 nm	abs 280.0 nm	260.0 nm / 280.0 nm	280.0 nm / 260.0 nm	Protein ug/ml	Nucleic Acid ug/ml
1	N	0.2022	0.1297	1.5591	0.6414	0.0000	808.8824
2	0.5	0.2110	0.1376	1.5328	0.6524	0.0000	843.8287
3	1	0.1591	0.1033	1.5401	0.6493	0.0000	636.2218
4	5 uM	0.1939	0.1280	1.5148	0.6602	0.0000	775.4238

To Page No.—

Witnessed & Understood by me, Yoshi

Date 6/5/01

Invented by J.S

Recorded by J.S

Date 12/22

Book No. 2 TITLE Growth assay

age No. To make Dose responce curve of MZ treatment,
① Cell count
② XTT assay was done.

ll : H460, H1299,
eatment : MZ (0 – 5 μM : cell count, 0 – 10 μM : XTT)
24, 48, 72 hours exposure

Cells were treated with MZ; 24 hours; Cell count; 0107-011101

H1299	0	0.01	0.05	0.1	0.5	5
1	3.38	3	2.88	3	2	0.75
2	2.88	3.38	2.75	3.38	2.5	0.63
3	2.88	2.75	3.5	2.5	1.88	0.63
Average	3.0467	3.0433	3.0433	2.96	2.127	0.67
SD	0.2357	0.259	0.3272	0.3604	0.268	0.0566
H460	0	0.01	0.05	0.1	0.5	5
1	5.5	6.38	6.63	6.13	2.13	0.38
2	6.63	6.25	6.25	4.25	1.75	0.5
3	7.88	7.13	6.25	5	1.25	0.5
Average	6.67	6.5867	6.3767	5.1267	1.71	0.46
SD	0.972	0.3878	0.1791	0.7727	0.36	0.0566

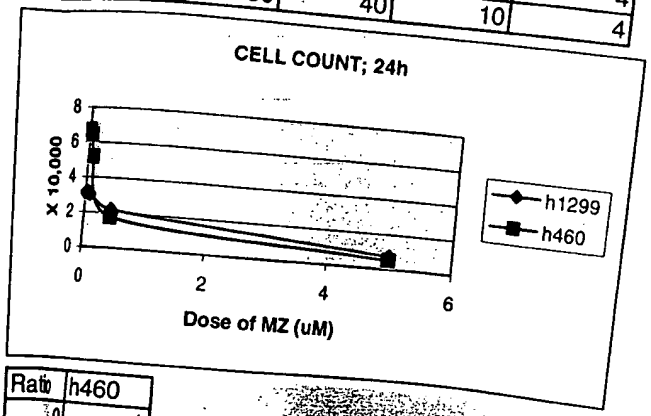
Average	0	0.01	0.05	0.1	0.5	5
h1299	3.0467	3.0433	3.0433	2.96	2.127	0.67
h460	6.67	6.5867	6.3767	5.1267	1.71	0.46
Ratio	0	0.25	0.5	1	2.5	10
h1299	1	0.9989	0.9989	0.9716	0.698	0.2199
h460	1	0.9875	0.956	0.7686	0.256	0.069

8

27	24	23	24	16	6
23	27	22	27	20	5
23	22	28	20	15	5

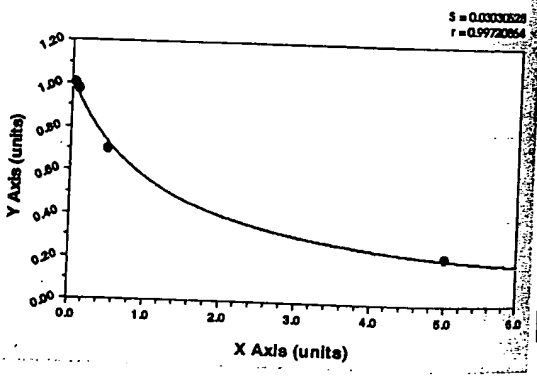
8

44	51	53	49	17	3
53	50	50	34	14	4
63	57	50	40	10	4



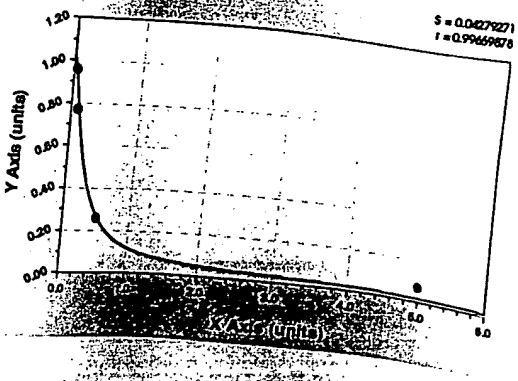
Ratio	h1299
0	1
0.01	0.9989
0.05	0.9989
0.1	0.9716
0.5	0.698
5	0.2199

IC20	0.33
IC50	1.29
IC80	5.2



Ratio	h460
0	1
0.01	0.9875
0.05	0.956
0.1	0.7686
0.5	0.2564
5	0.069

IC2	0.1
IC5	0.24
IC	0.63



To Page No. 11

& Understood by me,
Joshi

Date 6/5/01

Invented by JS

Recorded by J.S

Date 1/11/01

Project No. 3

Book No. 2 TITLE RT-PCR

Page No. VEGF - RTPCR after MZ treatment:

Cell: H1299, H460

Treatment: MZ Dose (0, 0.05, 0.1, 0.5, 1 μM) Time (0.1μM: 0, 1, 2, 6, 12, 24 h)

rid

les Method SaveClear Print Quit

file: A:\WORK_RES Method name: A:\RNA100

pe: General Ratio and Concentration Units: ug/ml

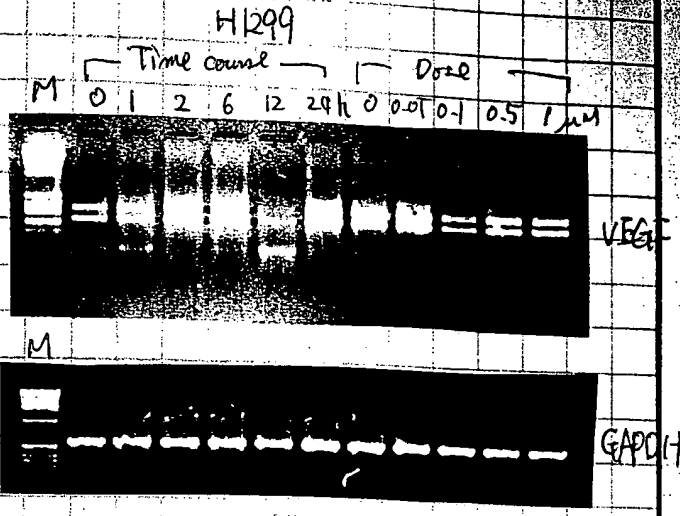
setup: VIEW Background Correction: [No]

device: None Concentration: [Yes]

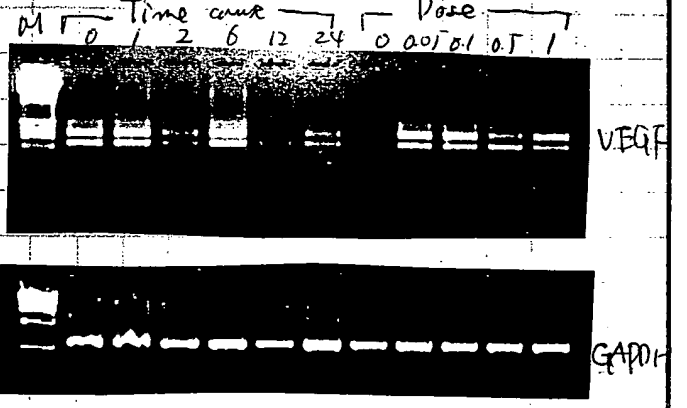
rage time: 0.50 sec Peak Pick: [No]

abs 260.0 nm	abs 280.0 nm	260.0 nm / 280.0 nm	280.0 nm / 260.0 nm	Protein ug/ml	Nucleic Acid ug/ml
0.1332	0.0801	1.6617	0.6018	0.0000	532.6299
0.5269	0.3118	1.6896	0.5918	0.0000	2107.5562
0.5868	0.3489	1.6822	0.5945	0.0000	2347.3457
0.6413	0.3719	1.7246	0.5799	0.0000	2565.3335
-0.3000	-0.3000	1.0000	1.0000	-0.0000	-1200.0000
✓ 0.5791	0.3421	1.6927	0.5908	0.0000	2316.2683
0.5624	0.3279	1.7151	0.5831	0.0000	2249.7939
1.0158	0.5580	1.8204	0.5493	0.0000	4063.1064
0.2683	0.1608	1.6678	0.5996	0.0000	1073.0630
0.5450	0.3118	1.7478	0.5722	0.0000	2180.0652
0.7140	0.4205	1.6980	0.5889	0.0000	2856.1829
0.5117	0.3033	1.6872	0.5927	0.0000	2046.7217
0.6982	0.4061	1.7192	0.5817	0.0000	2792.9055
0.6256	0.3685	1.6977	0.5890	0.0000	2502.4951
0.6685	0.3898	1.7152	0.5830	0.0000	2674.1338
0.4885	0.3012	1.6218	0.6166	0.0000	1954.0317
0.5343	0.3217	1.6609	0.6021	0.0000	2137.2446
0.5100	0.3112	1.6387	0.6102	0.0000	2040.1904
-0.3000	-0.3000	1.0000	1.0000	-0.0000	-1200.0000
0.5289	0.3211	1.6468	0.6072	0.0000	2115.4045
0.4389	0.2674	1.6415	0.6092	0.0000	1755.6592
0.5201	0.3133	1.6602	0.6023	0.0000	2080.4900
0.5445	0.3263	1.6689	0.5992	0.0000	2178.0122
0.5373	0.3215	1.6711	0.5984	0.0000	2149.1904

H1299



H460



Page No.

Read & Understood by me, Yoshi

Date 6/5/01

Invented by J.S

Recorded by J.S

1/12/01

Western Blot.

Book No.

No.

mple

PAGE : 10 %.

1st Ab : caspase 3 (×500)
caspase 9 (×500)
PARP (×500)
1hour, r.t

2nd Ab : Anti-mouse (PARP)
Anti-Rabbit (caspase)
1hour, r.t

Expoure : 5sec, 5min.

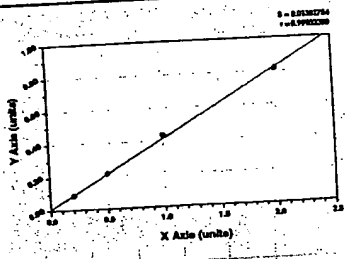
Re-probe : P21, BAX
β-actin, P53

Protein Concentration (Urea, BCA method); 010901

Albumin standard

Conc.(ug/ul)	0	0.2	0.5	1	2
OD	0	0.089	0.216	0.436	0.814

Conc.(ug/ul)	OD
0	0
0.2	0.089
0.5	0.216
1	0.436
2	0.814



Cell	H1299(24h)	50		
MZ(Um)	0	0.05	0.5	5
OD	2.184	2.257	1.893	1.763
OD(standard)	1.973	2.046	1.682	1.552
Protein(ug/ul)	4.82	5	4.11	3.79
50ug	10.37	10	12.17	13.19

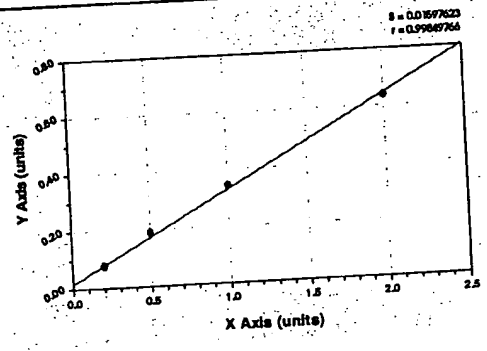
Cell	H460(24h)	50		
MZ(Um)	0	0.05	0.5	5
OD	2.536	2.49	2.279	2.381
OD(standard)	2.325	2.279	2.068	2.17
Protein(ug/ul)	5.69	5.57	5.06	5.31
50ug	8.79	8.98	9.88	9.42

Protein Concentration (Urea, BCA method); 011101

Albumin standard

Conc.(ug/ul)	0	0.2	0.5	1	2
OD	0	0.08	0.191	0.344	0.639

Conc.(ug/ul)	OD
0	0
0.2	0.08
0.5	0.191
1	0.344
2	0.639



Cell	A549(24h)		50	0.145		
Treatment	non-treat	UV(15J)	CDDP(10uM)	CDDP(50uM)	TAX(2uM)	TAX(10uM)
OD	0.952	0.693	0.992	0.815	0.944	0.823
OD(standarized)	0.807	0.548	0.847	0.67	0.799	0.678
Protein(ug/ul)	2.5	1.68	2.63	2.07	2.45	2.09
50ug	20	29.76	19.01	24.15	20.41	23.92

To Page No.

lessed & Understood by me, Yoshi	Date 6/5/01	Invented by J-S	Date 1/18/01
		Recorded by J-S	

Project No.____

Book No. 2 TITLE Western Blot.

e No.__

Sample : H460, H1299

Treatment : MZ Dose (0, 0.2, 0.5, 1 μM ; 24h)

Lysate : Cell fractionation

Volume : 25 μg

PAGE : 12.5 %

1st Ab : cytochrome C, COX IV (x500, x500) 1hour r.t

2nd Ab : Anti-mouse HRP (x2000)

Exposure : 15 sec and 5min

Re-probe : p53 & β-actin

To Page No.____

d & Understood by me,
yoshi

Date 6/5/01

Invented by J-S

Recorded by J-S

Date 2/10/01

Book No. 2 TITLE Western Blot.

Page No.

Sample : same as P.15.

Treatment : MR (0.1μM) → time course (0, 12, 24, 48h)
TAX (10nM), VNB (10nM)

Lysate :- SDS-Urea PAGE 12.5-1. 50μg

First Ab : caspase 3, caspase 9 1hour r.t. (×500)

2nd Ab : Anti-Rabbit HRP (×5000) 1h. r.t.

Exposure : 15sec

To Page No.

d & Understood by me,	Date	Invented by	Date
Joshi	6/5/01	J.S	2/20/01
		Recorded by J.S	

Page No.

Sample : H1299, H460

Treatment : MZ 0.5μM Time course (0, 30min, 2, 6, 12, 24, 48 h)
MZ 24h Dose (0, 0.2μM, 0.5μM, 1μM)

Method : PI 70% Etoh fixation

See FACS File (1)

To Page No.

Witnessed & Understood by me:	Date	Invented by	Date
yoshi	6/5/01	J.S	2/23
		Recorded by J.S	

Western Blot

Book No. 2

Page No. 18

Sample : same as P. 18.

PAGE : 12.5%.

1/25 1st Ab : caspase 8, Bid (x 500)

2nd Ab : Anti-Rabbit HRP (x5000)

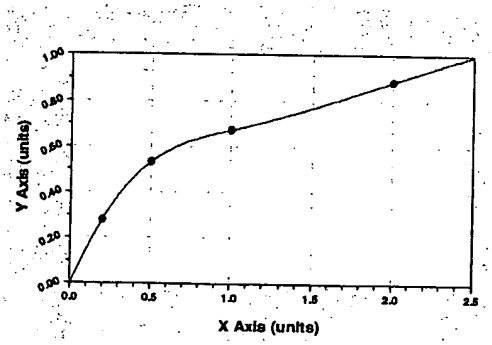
Exposure : 5min.

Protein Concentration (BF method); 022102

Albumin standard

Conc.(ug/ul)	0	0.2	0.5	1	2
OD	0	0.278	0.532	0.672	0.884

Conc.(ug/ul)	OD
0	0
0.2	0.278
0.5	0.532
1	0.672
2	0.884



Cell;H1299Cyto Fraction-DTT 50 0.293

Treatment	DMSO	MZ;0.2uM	MZ;0.2uM	MZ;1.0uM
OD	1.282	1.211	1.183	1.216
OD(standarized)	0.989	0.918	0.89	0.923
Protein(ug/ul)	2.46	2.14	2.03	2.17
50ug	20.33	23.36	24.63	23.04

Cell;H1299MT Fraction-DTT

Treatment	DMSO	MZ;0.2uM	MZ;0.2uM	MZ;1.0uM
OD	1.224	1.273	1.261	1.302
OD(standarized)	0.931	0.98	0.968	1.009
Protein(ug/ul)	2.2	2.42	2.36	2.55
50ug	22.73	20.66	21.19	19.61

Cell;H460Cyto ~~Urea~~-DTT

Treatment	DMSO	MZ;0.2uM	MZ;0.2uM	MZ;1.0uM	CDDP;100uM
OD	1.23	1.197	1.269	1.243	
OD(standarized)	0.937	0.904	0.976	0.95	
Protein(ug/ul)	2.23	2.09	2.4	2.28	
50ug	22.42	23.92	20.83	21.93	22

PAGE : 12.5%.

1/24 1st Ab : cytochrome C, coX IV (x500)

2nd Ab : Anti-mouse HRP (x2000)

Exposure : 5min.

Yuzhi 6/5/01 J.S 2/26/01

J.S

Project No. 3
Book No. 2 TITLE Western Blot.

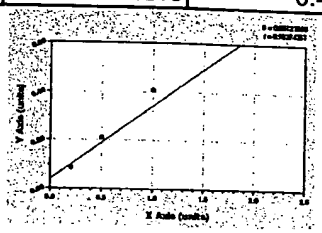
Sample

Protein Concentration (BCA method); 021501

Albumin standard

conc.(ug/ul)	0	0.2	0.5	1	2
	0	0.088	0.213	0.409	0.61

conc.(ug/ul)	OD
0	0
0.2	0.088
0.5	0.213
1	0.409
2	0.61



H460 SDS-DTT 50 0.114

treatment	0	UV;30min	MZ;30min	MZ;2h
	0.585	0.602	0.615	0.634
(standarized)	0.471	0.488	0.501	0.52
otein(ug/ul)	1.42	1.48	1.52	1.58
ng	35.21	33.78	32.89	31.65

H460 SDS-DTT 50 0.364

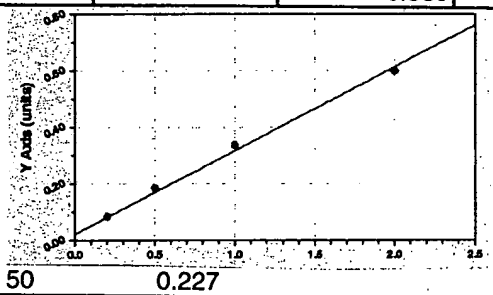
treatment	MZ;6h	MZ;12h	MZ;24h
	0.782	0.908	0.947
(standarized)	0.418	0.544	0.583
otein(ug/ul)	1.41	1.87	2.01
ng	35.46	26.74	24.88

ein Concentration (BCA method); 022101

umin standard

c.(ug/ul)	0	0.2	0.5	1	2
	0	0.086	0.185	0.339	0.602

c.(ug/ul)	OD
0	0
0.2	0.086
0.5	0.185
1	0.339
2	0.602



99 SDS-DTT 50 0.227

nt	DMSO;6h	MZ;0.2uM,6h	MZ;0.3uM,6h	MZ;0.4uM,6h	MZ;0.5uM,6h	MZ;1.0uM,6h
	0.997	0.938	0.876	0.971	0.932	0.929
larized)	0.77	0.711	0.649	0.744	0.705	0.702
g/ul)	2.52	2.32	2.11	2.43	2.3	2.29
	19.84	21.55	23.7	20.58	21.74	21.83

) SDS-DTT

nt	DMSO;6h	MZ;0.2uM,6h	MZ;0.3uM,6h	MZ;0.4uM,6h	MZ;0.5uM,6h	MZ;1.0uM,6h	MZ;6h'
	0.872	0.82	0.699	0.717	0.738	0.788	
larized)	0.645	0.593	0.472	0.49	0.511	0.561	
g/ul)	2.1	1.92	1.52	1.58	1.65	1.82	
	23.81	26.04	32.89	31.65	30.3	27.47	30

PAGE: 104. TBS system

Book No. 2

Witnessed by Date Recorded by

Yoshi 6/5/01 J.S 3/2-6

J.S

Page No. 20

1st Ab = ⓟ-Jun, ⓟ-ATF2 (3/2)
ⓟ-P38, ⓟ-P42/P44 ERK (3/3)
ⓟ-Bad, ⓟ-AKT (3/6)
) overnight, 4°C

2nd Ab = Anti Rabbit HRP (x5000)

Exposure = 30sec – 30min

FACS

Project No. 5
Book No. 2

Sample : H460

Treatment : DMSO (24h, 48h, 72h)
MZ 0.2μM (24h, 48h, 72h)
MZ 0.1μM (24h, 48h, 72h)
MZ 1.0μM (24h, 48h, 72h)
LDDP 50μM (24h, 48h, 72h)

See Facs File (2)

3/1/01

FACS

Project No. 5
Book No. 2

Sample : H299, H460, A549

Treatment : DMSO (12h, 24h, 48h)
MZ 0.2 μM (12h, 24h, 48h)
MZ 0.1 μM (12h, 24h, 48h)
MZ 1.0 μM (12h, 24h, 48h)

See FACS File (3)

3/16/01

Witnessed & Understood by me, Yoshi | Date 6/5/01 | Invented by J.S | Recorded by J-S | Date 3/2-6

Project No. 5

Book No. 2 TITLE Western Blot

Page No.

Re-probe

Sample : Same as P21. (H460, H299 dose & Time)

1st Ab

HIF-1α	(x250)	Cyclin G1	(x200)
P21	(x500)	cyclin A	(x500)
P27	(x200)		
GRP 78	(x200)		
GADD 153	(x200)		
BAX	(x500)		
Chk-1	(x200)		
Chk-2	(x200)		

1 hour r.t.

2nd Ab

Anti-mouse HRP	(HIF-1α, P21, cyclin A Chk-1)	x2000	
Anti-Rabbit HRP	(BAX, Chk-2 GADD153)	x5000	
Anti-goat HRP	(P27, GRP78 cyclin G1)	x10000	1hour r.t.

Exposure (30s, 3min, 30min)

Yoshi	6/5/01	J.S	3/19
		J-S	

DNA ladder

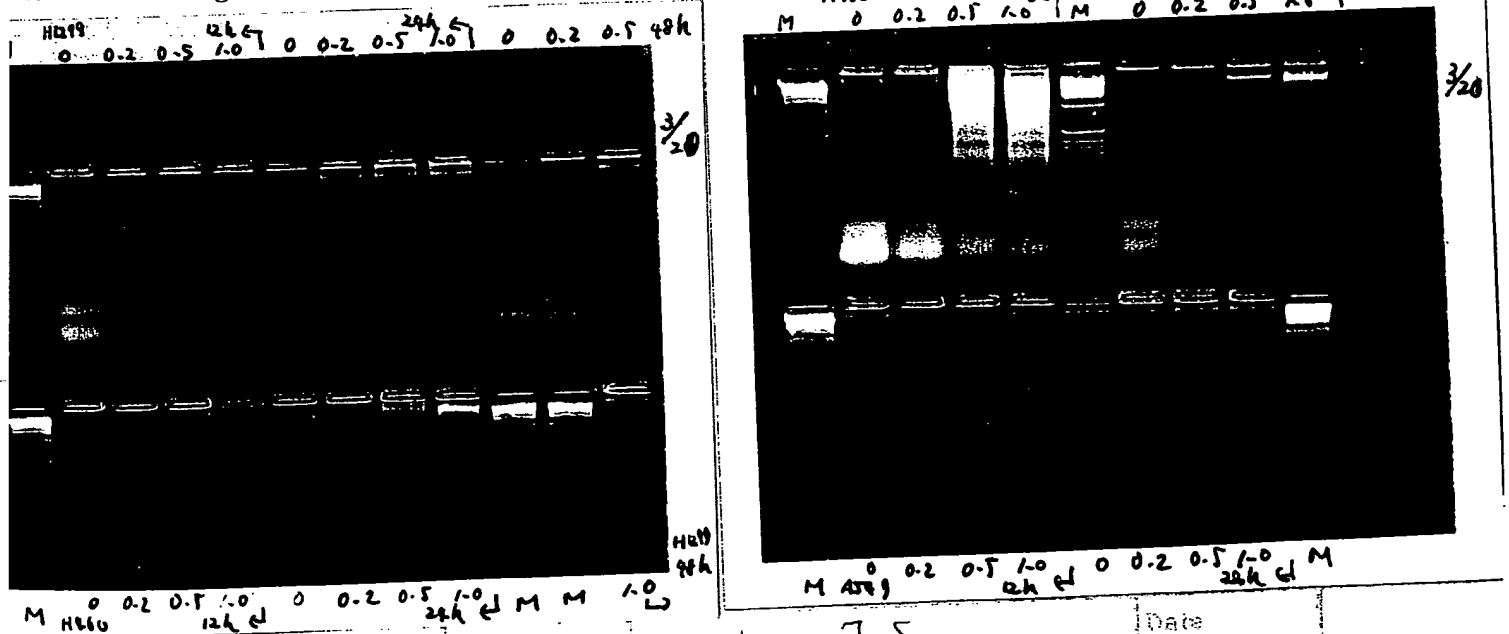
e No.—

Cell: H460, A549. H1299

Treatment: MZ dose: 0, 0.2, 0.5, 1 μM. Time: 12h, 24h, 48h

)NA fragmentation assay

The nucleosomal DNA degradation was analyzed as described previously with nodification. Briefly, 5 x 10^5 cells of each cell line were seeded in 100-mm culture dish and allowed to grow for 24 hours. The medium was then replaced with medium containing either DMSO or several doses of MZ. After 12, 24, 48 hours of incubation, both floating and attached cells were harvested and washed in cold PBS. The cell pellets were stored at –80°C until use. The pellets were lysed in 100 mm^3 of the lysis buffer (10 mM Tris (pH 7.4), 10mM EDTA (pH 8.0), 0.5% Triton X-100) and incubated for 10 min at 4°C. After centrifugation, the supernatants were incubated with 200 μg/ml of RNase A for 1 hour at 37°C. The supernatants were then incubated with 200 μg/ml of Proteinase K for 30 min at 50°C. After the incubation, DNA fragments were precipitated with 0.5 M of NaCl and 50% of isopropanol. The sample was loaded in 2% agarose gel and stained with ethidium bromide.



Boshi 6/5/01 J.S J.S Date 3/20

Project No. ___

Book No. 2 TITLE Western Blot

e No. Sample

Protein Concentration (BF method); 030701 MZ:0.5uM

Albumin standard

Conc.(ug/ul)	0	0.2	0.5	1	0.291
OD	0	0.368	0.658	0.804	0.924

Conc.(ug/ul)	OD
0	0
0.2	0.368
0.5	0.658
1	0.804
2	0.924

Cell;H1299Cyto Fraction-DTT 50 0.312

Treatment	0h	6h	12h	24h
OD	1.303	1.312	1.347	1.316
OD(standarized)	0.991	1	1.035	1.004
Protein(ug/ul)	2.5	2.5	2.5	2.5
50ug	20	20	20	20

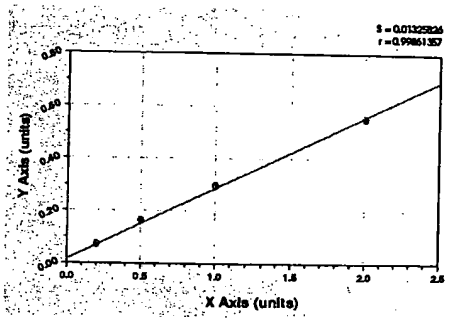
Cell;H460Cyto Urea-DTT

Treatment	0h	6h	12h	24h
OD	1.255	1.195	1.218	1.215
OD(standarized)	0.943	0.883	0.906	0.903
Protein(ug/ul)	2.18	1.62	1.78	1.78
50ug	22.94	30.86	28.09	28.09

Concentration (BCA method); 032201

n standard 0.092

ıg/ul)	OD
0	0
0.2	0.074
0.5	0.164
1	0.297
2	0.554



299, 48h Urea-DTT 50 0.139

ent	DMSO+P53(1moi)	MZ;0.1uM+P53(1moi)	MZ;0.2uM+P53(1moi)	MZ;0.2uM+GFP(1moi)	MZ;0.5uM	P53(10moi)
	1.232	1.089	0.957	1.326	0.995	0.486
darized)	1.093	0.95	0.818	1.187	0.856	0.347
ug/ul)	3.95	3.42	2.94	4.29	3.08	1.21
	12.66	14.62	17.01	11.66	16.23	41.32

60, 48h Urea-DTT

ent	DMSO+P53(1moi)	MZ;0.1uM+P53(1moi)	MZ;0.2uM+P53(1moi)	MZ;0.2uM+GFP(1moi)	MZ;0.5uM	P53(10moi)
	1.728	1.316	1.349	1.177	0.718	1.318
darized)	1.589	1.177	1.21	1.038	0.579	1.179
ug/ul)	5.77	4.26	4.38	3.75	2.06	4.26
	8.67	11.74	11.42	13.33	24.27	11.74

To Page No. 25

Date 2/21/02 J.S

Recorded J.S 3/29-31/01

Page No. 29

1st Ab : Cytochrome C (x500) → Fraction (3/7) } (3/29)
Caspase 3 (x500) → Urea (3/22) }

~~Re-probe : β-actin & P53 (3/31)~~

PARP (x1000)
HIF1-α (x200) } Urea (3/22) (3/30)
P53 + β-actin

Re-probe : β-actin & P53

To Page No.

Read & Understood by me.	Date	Invented by	Date
Yoshi	2/21/02	J.S	3/29/31/01
		Recorded by J.S	

Book No. 2 TITLE Western Blot

Page No.

Sample : Fraction sample H1299, H460 → (3/7)
Urea-SDS sample H1299, H460 ↴

Protein Concentration (BCA method); 022701
Albumin standard

Conc.(ug/ul)	0	0.2	0.5	1	2
OD	0	0.094	0.208	0.336	0.626

Conc.(ug/ul)	OD
0	0
0.2	0.094
0.5	0.208
1	0.336
2	0.626

Cell;H1299 48h Urea-DTT 50 0.236

Treatment	DMSO	MZ;0.2uM	MZ;0.5uM	MZ;1.0uM
OD	1.957	1.702	1.153	1.231
OD(standarized)	1.721	1.466	0.917	0.995
Protein(ug/ul)	5.57	4.73	2.93	3.18
50ug	8.98	10.57	17.06	15.72

Cell;H460 48h Urea-DTT

Treatment	DMSO	MZ;0.2uM	MZ;0.5uM	MZ;1.0uM
OD	1.752	1.554	0.928	0.785
OD(standarized)	1.516	1.318	0.692	0.549
Protein(ug/ul)	4.9	4.25	2.19	1.71
50ug	10.2	11.76	22.83	29.24

Ab : cytochrome C (x1000) → (3/7)
Caspase 3 (x500)
Caspase 9 (x500) } (2/7) } 4/3
Caspase 8 (x500)

probe β-actin → (cytochrome C), (caspase 3)
p53 → caspase 9 } 4/4
p21 → caspase 8

To Page No.

Date 2/21/02 J.S 4/3-4/01
J-S

Cyto	Fraction-DTT	50	0.018	
	DMSO	MZ;0.2uM	MZ;0.5uM	MZ;1.0uM
	1.017	1	1	1.035
arized)	0.999	0.982	0.982	1.017
/ul)	1.98	1.89	1.89	2.07
	25.25	26.46	26.46	24.15

MT	Fraction-DTT			
	DMSO	MZ;0.2uM	MZ;0.5uM	MZ;1.0uM
	1.1	1.12	1.01	1.01
arized)	1.082	1.102	0.992	0.992
/ul)	2.4	2.49	1.94	1.94
	20.83	20.08	25.77	25.77

NUC	Fraction-DTT			
	DMSO	MZ;0.2uM	MZ;0.5uM	MZ;1.0uM
	1.242	1.229	1.308	1.256
arized)	1.224	1.211	1:29	1.238
/ul)	2.99	2.95	3.21	3.04
	16.72	16.95	15.58	16.45

Cyto	Fraction-DTT	50	0.018			
	DMSO	MZ;0.2uM	MZ;0.2uM vnb	vnb	MZ; 0.2uM, 0h	MZ; 0.2uM, 1
	0.947	0.969	0.93	0.925	0.938	0.91
rized)	0.929	0.951	0.912	0.907	0.92	0.
ul)	1.63	1.73	1.54	1.52	1.58	1.4
	30.67	28.9	32.47	32.89	31.65	33.5

MT	Fraction-DTT					
	DMSO	MZ;0.2uM	MZ;0.2uM vnb	vnb	MZ; 0.2uM, 0h	MZ; 0.2uM,
	0.9	0.975	1.007	0.967	0.913	0.9
rized)	0.882	0.957	0.989	0.949	0.895	0.9
ul)	1.41	1.76	1.93	1.72	1.47	1.
	35.46	28.41	25.91	29.07	34.01	30.

NUC	Fraction-DTT					
	DMSO	MZ;0.2uM	MZ;0.2uM vnb	vnb	MZ; 0.2uM, 0h	MZ; 0.2uM,
	1.237	1.242	1.221	1.232	1.159	1.2
ized)	1.219	1.224	1.203	1.214	1.141	1.2
ul)	2.97	2.99	2.92	2.96	2.67	2.
	16.84	16.72	17.12	16.89	18.73	16.

t: Ab : Cytochrome C (x1000) → (H460 Frac - MZ+UNB - Cytosolic
H1299 Frac - MZ+P53 - Cytosolic

Caspase 3, caspase 9 (x500) → A549 Urea-SDS

To Page No.

Yoshi 2/21/02 Recorded by J.S J.S 4/14

Page No.

Sample : (4/10)

1st Ab : BAX (x1000) → (H460 Frac - MZ+VNB - mitochondrial
H1299 Frac - MZ+P53 - mitochondrial

Caspase 3, caspase 9 H460 Urea - DTT → P53+MZ

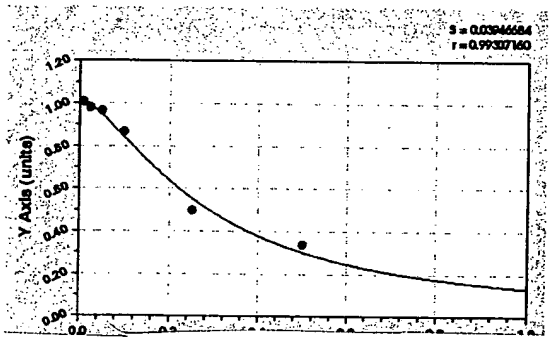
Re-probe : P53 (x2000) → A549 Urea-SDS, H460-HitoF
P21 (x1000) → A549 Urea-SDS
B-actin → H1299 - cyto F, H460 - cyto F., H460 - Urea-DTT P53+MZ

XTT assay Project 5/ Book No 2

41901 XTT-48h h1299 MZ-dose

blank	0(uM)	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
0.196	1.176	1.146	1.137	1.154	1.024	0.55	0.381	0.363	0.341	0.323	0.352
0.19	1.138	1.168	1.19	1.156	1.037	0.569	0.41	0.361	0.357	0.354	0.341
0.19	1.201	1.193	1.16	1.101	0.977	0.566	0.412	0.369	0.372	0.348	0.308
0.193	1.187	1.185	1.108	1.111	1.066	0.592	0.398	0.419	0.344	0.363	0.361
	1.231	1.186	1.089	1.07	1.008	0.6	0.426	0.368	0.354	0.345	0.353
	1.135	1.188	1.141	1.12	1.009	0.572	0.387	0.356	0.333	0.319	0.363
	1.121	1.185	1.135	1.159	1.01	0.607	0.402	0.343	0.355	0.323	0.323
	1.06	1.123	1.098	1.103	0.957	0.595	0.37	0.353	0.332	0.326	0.308
average	1.1561	1.1718	1.1323	1.122	1.011	0.58138	0.3983	0.3665	0.3485	0.337625	0.338625
ratio	1	1.01	0.98	0.97	0.87	0.5	0.34	0.32	0.3	0.29	0.29

0	1
0.01	1.01
0.025	0.98
0.05	0.97
0.1	0.87
0.25	0.5
0.5	0.34
1	0.32
2.5	0.3
5	0.29



IC20	0.12
IC50	0.288
IC80	0.72

Witnessed & Understood by me: Zozhi Date 2/21/02 Invented by J. S Recorded by J. S Date 4/19/01

Project No. 5

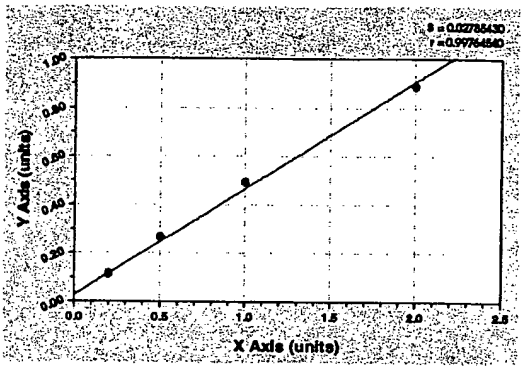
Book No. 2 TITLE Western Blot

age No. Sample : H460 ①

ı Concentration (BCA method); 042101

n standard

ug/ul)	OD
0	0
0.2	0.116
0.5	0.267
1	0.496
2	0.888



549 Urea-DTT 50 0.092 x5

ent	MZ; 5uM, 0h	MZ; 5uM, 12h	MZ; 5uM, 24h	MZ; 5uM, 48h	DMSO;48h	1uM;48h	5uM;48h	10uM;48h
	0.573	0.64	0.628	0.446	0.536	0.448	0.415	0.44
ndarized)	0.481	0.548	0.536	0.354	0.444	0.356	0.323	0.348
(ug/ul)	1.03	1.18	1.16	0.74	0.95	0.75	0.67	0.73
(ug/ul) True	5.15	5.9	5.8	3.7	4.75	3.75	3.35	3.65
	9.71	8.47	8.62	13.51	10.53	13.33	14.93	13.7

460 ① SDS-DTT 50 0.334 x5

ent	UV;2h	CDDP(50uM)	MZ;0.5uM, 0h	12h	24h	48h	DMSO;48h	MZ;0.2uM	M
	0.726	0.67	0.752	0.644	0.549	0.658	0.918	0.909	
ndarized)	0.392	0.336	0.418	0.31	0.215	0.324	0.584	0.575	
ug/ul)	0.83	0.7	0.89	0.64	0.42	0.67	1.26	1.24	
ug/ul) True	4.15	3.5	4.45	3.2	2.1	3.35	6.3	6.2	
	12.05	14.29	11.24	15.63	23.81	14.93	7.94	8.06	

99 SDS-DTT 50 0.147 x5

nt	Co(100uM)	Aniso;50nM	UV;2h	MZ; 0.5uM	12h	24h	48h	DMSO;	0.2uM	0.5uM	1uM
	0.394	0.39	0.453	0.297	0.308	0.316	0.407	0.761	0.663	0.525	0.467
dar	0.247	0.243	0.306	0.15	0.161	0.169	0.26	0.614	0.516	0.378	0.32
g/u	0.84	0.82	1.05	0.48	0.52	0.55	0.88	2.17	1.81	1.31	1.1
g/u	4.2	4.1	5.25	2.4	2.6	2.75	4.4	10.85	9.05	6.55	5.5
	11.9	12.2	9.52	20.83	19.23	18.18	11.36	4.61	5.52	7.63	9.09

9①SDS-DTT 50 0.147 x5

it	Co(100uM)	Aniso;50nM	UV;2h	CDDP(50	MZ; 0h,	12h	24h	48h	DMSO	0.5uM	1uM	2
	0.427	0.374	0.41	0.241	0.366	0.3	0.303	0.341	0.442	0.36	0.321	
lar	0.28	0.227	0.263	0.094	0.219	0.153	0.156	0.194	0.295	0.213	0.174	
g/u	0.96	0.76	0.89	0.28	0.73	0.49	0.5	0.64	1.01	0.71	0.57	
g/u	4.8	3.8	4.45	1.4	3.65	2.45	2.5	3.2	5.05	3.55	2.85	
	10.42	13.16	11.24	35.71	13.7	20.41	20	15.63	9.9	14.08	17.54	

② SDS-DTT 50 0.147 x5

t	Co(100uM)	Aniso;50nM	UV;2h	MZ; 0.5uM	12h	24h	48h	DMSO;	0.2uM	0.5uM	1uM	N
	0.576	0.393	0.416	0.401	0.263	0.297	0.415	0.726	0.79	0.539	0.434	
ar	0.429	0.246	0.269	0.254	0.116	0.15	0.268	0.579	0.643	0.392	0.287	
g/u	1.5	0.83	0.92	0.86	0.36	0.48	0.88	2.04	2.27	1.36	0.98	
g/u	7.5	4.15	4.6	4.3	1.8	2.4	4.4	10.2	11.35	6.8	4.9	
	6.67	12.05	10.87	11.63	27.78	20.83	11.36	4.9	4.41	7.35	10.2	

0.31

se I & Understood by me

Yoshi

Date 2/21/02

Invented by J. S

Recorded by J S

Date 4/26-27

Book No. 2 TITLE XTT assay

Page No. Growth inhibition assay.

Design

day 0
Seed cells
1000/well (H460, H1299)
2000/well (A549)

day 1
Treatment MZ (0 ~ 10μM)
with or without inhibitory agents
1) DMSO – control
2) PDTC – JNK inhibitor
3) SB202190 } P38 inhibitor
4) SB203500 }
5) Ly294002 – ~~AKT~~ PI-3k inhibitor
6) UO126 – MEK1 inhibitor

day 3
XTT

A549

data 6/6/~~97~~ 01 A549 DMSO/PDTC

1	2	3	4	5	6	7	8	9	10	11	12
	1.301	1.319	1.329	1.266	1.218	0.884	0.778	0.681	0.69	0.714	0.66
	1.278	1.328	1.275	1.264	1.184	0.888	0.784	0.683	0.717	0.665	0.642
	1.348	1.27	1.264	1.294	1.231	0.9	0.782	0.72	0.699	0.694	0.658
	1.308	1.301	1.235	1.279	1.235	0.977	0.907	0.677	0.652	0.657	0.607
202	1.301	1.294	1.179	1.202	1.163	0.821	0.766	0.71	0.695	0.673	0.661
201	0.276	0.29	0.295	0.272	0.297	0.284	0.323	0.301	0.32	0.316	0.34
208	0.21	0.211	0.21	0.206	0.211	0.211	0.214	0.21	0.213	0.216	0.222
216	0.223	0.221	0.225	0.215	0.219	0.219	0.218	0.219	0.22	0.221	0.225

575

MZ(uM)

%)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
0	1.09425	1.11225	1.12225	1.05925	1.01125	0.67725	0.57125	0.47425	0.48325	0.50725	0.45325
08	1.07125	1.12125	1.06825	1.05725	0.97725	0.68125	0.57725	0.47625	0.51025	0.45825	0.43525
08	1.14125	1.06325	1.05725	1.08725	1.02425	0.69325	0.57525	0.51325	0.49225	0.48725	0.45125
4	1.10125	1.09425	1.02825	1.07225	1.02825	0.77025	0.70025	0.47025	0.44525	0.45025	0.40025

M)

0	1.09425	1.08725	0.97225	0.99525	0.95625	0.61425	0.55925	0.50325	0.48825	0.46625	0.45425
1	0.06925	0.08325	0.08825	0.06525	0.09025	0.07725	0.11625	0.09425	0.11325	0.10925	0.13325
25	0.00325	0.00425	0.00325	0.0007	0.00425	0.00425	0.00725	0.00325	0.00625	0.00925	0.01525
00	0.01625	0.01425	0.01825	0.00825	0.01225	0.01225	0.01125	0.01225	0.01325	0.01425	0.01825

MZ(uM)

6)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
0	1	1.016	1.026	0.968	0.924	0.619	0.522	0.433	0.442	0.464	0.414
8	0.979	1.025	0.976	0.966	0.893	0.623	0.528	0.435	0.466	0.419	0.398
8	1.043	0.972	0.966	0.994	0.936	0.634	0.526	0.469	0.45	0.445	0.412
4	1.006	1	0.94	0.98	0.94	0.704	0.64	0.43	0.407	0.411	0.366
1)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
0	1	0.994	0.889	0.91	0.874	0.561	0.511	0.46	0.446	0.426	0.415
1	0.063	0.076	0.081	0.06	0.082	0.071	0.106	0.086	0.103	0.1	0.122
5	0.003	0.004	0.003	0.001	0.004	0.004	0.007	0.003	0.006	0.008	0.014
0	0.015	0.013	0.017	0.008	0.011	0.011	0.01	0.011	0.012	0.013	0.017

39

Understood by me.	Date	Invented by	Date
yoshi	2/21/02	J.S	Jan/6
		Recorded by J.S	

E XTT assay Project No. 3 Book No. 2 3

m Page No. 38

H460

Row c : 6/6/~~97~~ 0/ H460 DMSO/PDTC

	2	3	4	5	6	7	8	9	10	11	12
	1.738	1.468	1.558	1.539	1.513	0.614	0.495	0.385	0.428	0.414	0.369
	1.591	1.526	1.456	1.467	1.408	0.564	0.458	0.389	0.426	0.422	0.377
	1.591	1.596	1.579	1.585	1.51	0.571	0.49	0.4	0.418	0.409	0.369
	1.495	1.511	1.579	1.505	1.529	0.604	0.475	0.408	0.397	0.422	0.372
	1.554	1.555	1.611	1.557	1.51	0.636	0.482	0.397	0.42	0.429	0.369
	0.211	0.214	0.209	0.209	0.208	0.212	0.207	0.212	0.208	0.216	0.216
6	0.225	0.22	0.221	0.214	0.218	0.214	0.218	0.213	0.219	0.216	0.216
23	0.229	0.23	0.228	0.227	0.226	0.226	0.225	0.224	0.223	0.218	0.222

225

MZ(uM)

S O(%)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
0	1.516	1.246	1.336	1.317	1.291	0.392	0.273	0.163	0.206	0.192	0.147
.008	1.369	1.304	1.234	1.245	1.186	0.342	0.236	0.167	0.204	0.2	0.155
0.08	1.369	1.374	1.357	1.363	1.288	0.349	0.268	0.178	0.196	0.187	0.147
0.4	1.273	1.289	1.357	1.283	1.307	0.382	0.253	0.186	0.175	0.2	0.15

SI (uM)											
0	1.332	1.333	1.389	1.335	1.288	0.414	0.26	0.175	0.198	0.207	0.147
1	0	0	0	0	0	0	0	0	0	0	0
25	0.003	0	0	0	0	0	0	0	0	0	0
100	0.007	0.008	0.006	0.005	0.004	0.004	0.003	0.002	0.001	0	0

SE MZ(uM)

%)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
0	1	0.822	0.881	0.869	0.852	0.259	0.18	0.108	0.136	0.127	0.097
08	0.903	0.86	0.814	0.821	0.782	0.226	0.156	0.11	0.135	0.132	0.102
08	0.903	0.906	0.895	0.899	0.85	0.23	0.177	0.117	0.129	0.123	0.097
SB: .4	0.84	0.85	0.895	0.846	0.862	0.252	0.167	0.123	0.115	0.132	0.099
M	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
0	1	1.001	1.043	1.002	0.967	0.311	0.195	0.131	0.149	0.155	0.11
1	0	0	0	0	0	0	0	0	0	0	0
5	0.002	0	0	0	0	0	0	0	0	0	0
0	0.005	0.006	0.005	0.004	0.003	0.003	0.002	0.002	0.001	0	0

40

esearch Notebook

yoshi 2/21/02 Witnessed by J.S Jun/6

XTT assay

Page No. To investigate MZ activity to pancreas cancer cells:

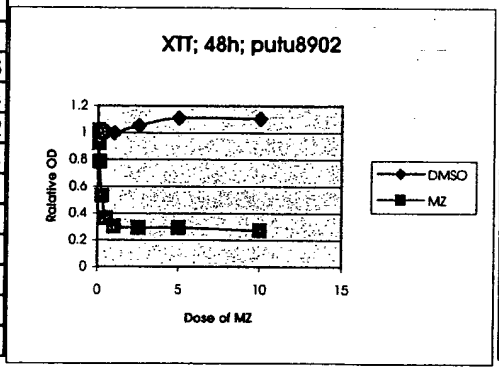
Design : day 0 — Seed cells 2000/well
day 1 — Treatment with DMSO or MZ (0-10μM)
day 3 — XTT assay

Sample : Putu 8902 , Putu8988T , Putu89885

Row data 6/7/01 PUTU8902

Row	1	2	3	4	5	6	7	8	9	10	11	12
	0.169	0.543	0.551	0.518	0.547	0.583	0.562	0.555	0.562	0.599	0.613	0.603
	0.165	0.55	0.547	0.555	0.561	0.567	0.545	0.573	0.544	0.582	0.577	0.579
	0.17	0.543	0.547	0.558	0.567	0.515	0.526	0.528	0.534	0.552	0.576	0.581
	0.179	0.541	0.542	0.556	0.494	0.529	0.523	0.533	0.535	0.521	0.575	0.57
		0.54	0.546	0.538	0.487	0.43	0.357	0.297	0.28	0.273	0.281	0.273
		0.547	0.543	0.548	0.492	0.457	0.356	0.308	0.275	0.279	0.278	0.265
		0.545	0.557	0.542	0.534	0.48	0.379	0.313	0.288	0.285	0.282	0.267
	0.17075	0.542	0.555	0.537	0.541	0.485	0.382	0.305	0.291	0.282	0.279	0.28
DMSO(%)	0	0.0001	0.00025	0.0005	0.001	0.0025	0.005	0.01	0.025	0.05	0.1	
	0.37225	0.38025	0.34725	0.37625	0.41225	0.39125	0.38425	0.39125	0.42825	0.44225	0.43225	
	0.37925	0.37625	0.38425	0.39025	0.39625	0.37425	0.40225	0.37325	0.41125	0.40625	0.40825	
	0.37225	0.37625	0.38725	0.39625	0.34425	0.35525	0.35725	0.36325	0.38125	0.40525	0.41025	
	0.37025	0.37125	0.38525	0.32325	0.35825	0.35225	0.36225	0.36425	0.35025	0.40425	0.39925	
AVERAGI	0.3735	0.376	0.376	0.3715	0.37775	0.36825	0.3765	0.373	0.39275	0.4145	0.4125	
RATIO	1	1.007	1.007	0.995	1.011	0.986	1.008	0.999	1.052	1.11	1.104	
MZ(uM)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10	
	0.36925	0.37525	0.36725	0.31625	0.25925	0.18625	0.12625	0.10925	0.10225	0.11025	0.10225	
	0.37625	0.37225	0.37725	0.32125	0.28625	0.18525	0.13725	0.10425	0.10825	0.10725	0.09425	
	0.37425	0.38625	0.37125	0.36325	0.30925	0.20825	0.14225	0.11725	0.11425	0.11125	0.09625	
	0.37125	0.38425	0.36625	0.37025	0.31425	0.21125	0.13425	0.12025	0.11125	0.10825	0.10925	
AVERAGI	0.37275	0.3795	0.3705	0.34275	0.29225	0.19775	0.135	0.11275	0.109	0.10925	0.1005	
RATIO	1	1.018	0.994	0.92	0.784	0.531	0.362	0.302	0.292	0.293	0.27	

	DMSO	MZ
0	1	1
0.01	1.007	1.018
0.025	1.007	0.994
0.05	0.995	0.92
0.1	1.011	0.784
0.25	0.986	0.531
0.5	1.008	0.362
1	0.999	0.302
2.5	1.052	0.292
5	1.11	0.293
10	1.104	0.27



	MZ
0	1
0.01	1.018
0.025	0.994
0.05	0.92
0.1	0.784
0.25	0.531
0.5	0.362
1	0.302
2.5	0.292
5	0.293
10	0.27

IC20	0.09
IC50	0.33
IC80	1.25

To Page No.

Read & understood by: Yoshi
Date: 2/21/02
Witnessed by: J. S
Recorded by: J.S
Date: Jun/7

XTT assay

Page No.

Design : Same as page 41

Sample : putu 89881 , putu89885 , MCF-7 , Putu8902 .

data	6/16/01 PUTU8988t PUTU8902											
1	2	3	4	5	6	7	8	9	10	11	12	0.4275
0.23	0.555	0.574	0.575	0.582	0.579	0.526	0.429	0.387	0.359	0.336	0.334	0.4485
0.224	0.573	0.571	0.584	0.596	0.587	0.529	0.437	0.385	0.352	0.346	0.334	0.4105
0.227	0.588	0.576	0.603	0.607	0.583	0.521	0.441	0.379	0.371	0.343	0.335	0.4145
0.227	0.577	0.601	0.586	0.587	0.572	0.552	0.434	0.388	0.355	0.348	0.332	0.42525
	0.753	0.733	0.739	0.752	0.711	0.548	0.439	0.369	0.381	0.368	0.362	0.74
	0.71	0.754	0.743	0.732	0.688	0.537	0.424	0.377	0.373	0.367	0.355	
	0.7	0.745	0.753	0.733	0.689	0.556	0.427	0.366	0.37	0.377	0.358	10
0.227	0.689	0.735	0.744	0.71	0.673	0.537	0.426	0.363	0.361	0.364	0.352	0.5745
												0.6185

U8988t												0.6095
uM)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10	0.6075
	0.328	0.347	0.348	0.355	0.352	0.299	0.202	0.16	0.132	0.109	0.107	0.6025
	0.346	0.344	0.357	0.369	0.36	0.302	0.21	0.158	0.125	0.119	0.107	0.74
	0.361	0.349	0.376	0.38	0.356	0.294	0.214	0.152	0.144	0.116	0.108	
	0.35	0.374	0.359	0.36	0.345	0.325	0.207	0.161	0.128	0.121	0.105	
ERAG	0.34625	0.3535	0.36	0.366	0.35325	0.305	0.20825	0.15775	0.13225	0.11625	0.10675	
TIO	1	1.02	1.04	1.06	1.02	0.88	0.6	0.46	0.38	0.34	0.31	

TU8902											
(uM)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
	0.526	0.506	0.512	0.525	0.484	0.321	0.212	0.142	0.154	0.141	0.135
	0.483	0.527	0.516	0.505	0.461	0.31	0.197	0.15	0.146	0.14	0.128
	0.473	0.518	0.526	0.506	0.462	0.329	0.2	0.139	0.143	0.15	0.131
	0.462	0.508	0.517	0.483	0.446	0.31	0.199	0.136	0.134	0.137	0.125
ERAG	0.486	0.51475	0.51775	0.50475	0.46325	0.3175	0.202	0.14175	0.14425	0.142	0.12975
TIO	1	1.06	1.07	1.04	0.95	0.65	0.42	0.29	0.3	0.29	0.27

w	data	6/16/01 PUTU81885 MCF-7									
1	2	3	4	5	6	7	8	9	10	11	12
0.232	0.805	0.775	0.793	0.778	0.701	0.695	0.622	0.584	0.606	0.639	0.657
0.227	0.813	0.765	0.75	0.803	0.736	0.718	0.62	0.588	0.603	0.63	0.678
0.228	0.805	0.78	0.801	0.805	0.75	0.72	0.599	0.579	0.651	0.68	0.64
0.231	0.802	0.809	0.818	0.823	0.777	0.697	0.568	0.616	0.659	0.638	0.644
	1.05	1.1	1.02	1.031	0.813	0.834	0.803	0.781	0.852	0.844	0.804
	1.026	1.03	1.05	1.047	1.045	0.897	0.86	0.812	0.808	0.835	0.848
	1.042	1.053	1.068	1.027	1.067	0.891	0.867	0.864	0.838	0.814	0.839
0.2295	1.059	1.07	1.037	1.054	1.063	0.848	0.797	0.808	0.777	0.796	0.837

JTU81885											
IZ(uM)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10

Page No.

Witnessed & Understood by: Yoshi 2/21/02

Recorded by: J.S. J-S.

June/16/01

m Page No. To quantify the efficacy of tubulin polymelyzation / depolymelyzation by MZ, we developed modified tubulin polimelyzation assay.

Design

day 0
Seed cells in 12well
(5×10^4/well/2ml) x3

day 1
~~Treatment with~~ Lysis with
- DMSO
- MZ (0.5 μM)
- NCZ (2.5 μM)
- TAX (10nM)

for 5min
~~for 3hours~~

→ Assay. (Immunoblot with α-tubulin Ab)

⇒ All treatment showd soluble tubulin expression in blot!

Immunostaing : α-tubulin
Sample : H460, A549, H1299

Design

day 0 (1×10^4 cells/well, 2x chamber slide) → day 1 Treatment → day 2 Stain

Treatment
- DMSO
- MZ : 0.5μM
- NCZ : 2.5μM
- TAX : 10nM

1) Remove medium and wash with cold PBS
2) Fix cells in 4% Paraformaldehyde for 10min at 4°C
3) Wash in cold PBS
4) Permeabilizes cells with 1ml solution containg Triton-X 100 0.1% and Sodium Citrate 0.1% for 2min.
5) Wash in cold PBS twice
6) Blocking for 20min
7) do same as 5)
8) Primary Ab reaction for 1hour
9) do same as 5)
10) Secondary Ab reaction for 1hour (with DAPI 0.5μg/ml)
11) do same as 5)
12) Air dry → mount

To Page No. 51

Book No. 2 TITLE Tubulin polymerization assay.

Page No.

Design: changed concentration of treating agent.

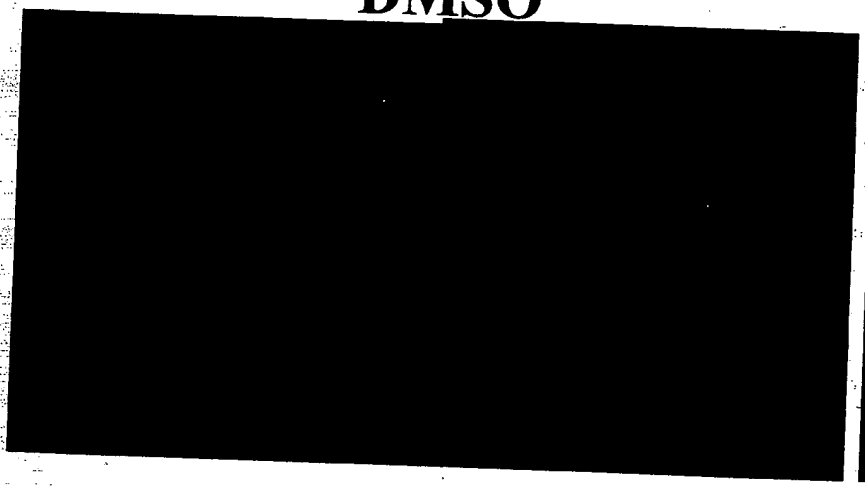
① MZ 0.5 μM
NCZ 2.5 μM
TAX 10 nM

② MZ 5 μM
NCZ 25 μM
TAX · 100 nM

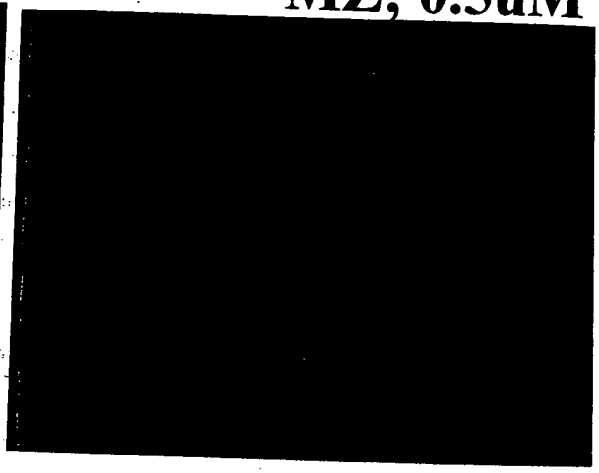
for 3 hours

Tubulin staining in A549 CELL

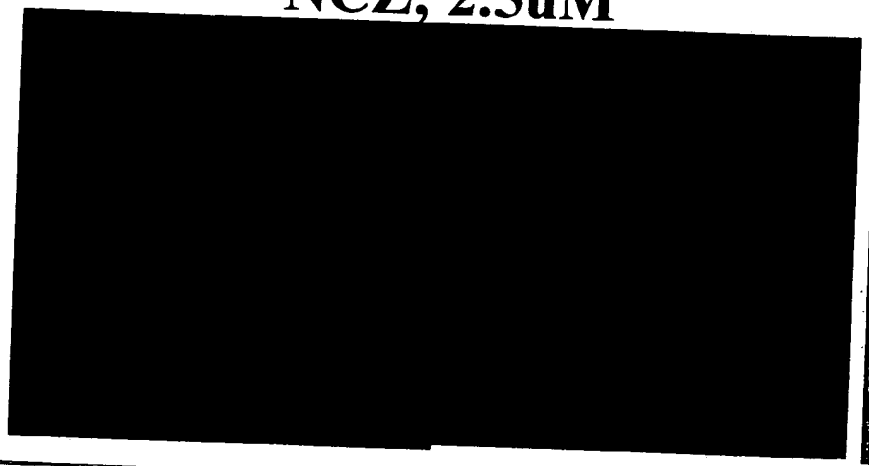
DMSO



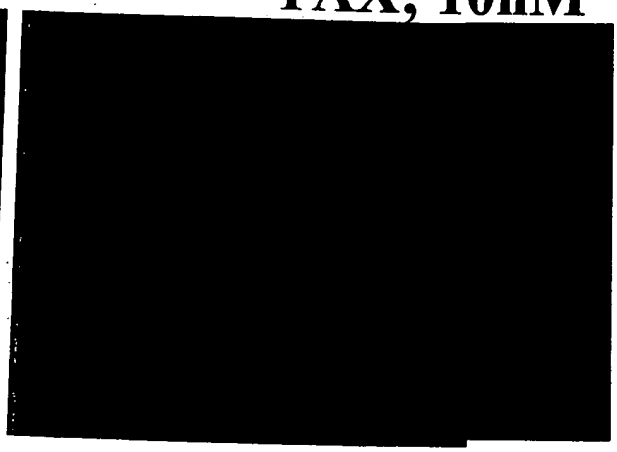
MZ; 0.5uM



NCZ; 2.5uM



TAX; 10nM



& Understood by me,

Date 2/21/12

Invented by J.D

Recorded by J.S

Date July/26-28

Book No. 2 TITLE Tubulin polimeryzation assay.

age No.

Design

day 0
Seed cells in 12well plate
5×10^5/well

day 1
Treatment with
- DMSO
- MZ 0.1μM
- NCZ ~~0.1μM~~
- TAX ~~10~~ 3 nM

for 3 hours → assay.

Lysis buffer with 4μg/ml TAX. (7/28)

this time ⇩ It is necessary to reduce of TAX.

H1299 → 4μg/ml ⇒ 2 μg/ml

H460
A549 > 4μg/ml ⇒ 0.5μg/ml

H1299 ⇒ unstable

2-4μg/ml of TAX must be needed

	DMSO		MZ		NCZ		TAX	
	S	P	S	P	S	P	S	P
H460								
H1299								
A549								

too much

OIC.

Read & Understood by	Date	Witnessed by	Date
Yoshi	2/21/02	J.S	8/1/01
		J.S	

Measurement of soluble and assembled tubulin.

Measurement of soluble (depolymerized) and assembled (polymerized) tubulin was analyzed as described previously with some modifications. Briefly, cells were grown in triplicate wells of a 12-well dish for 24 hours and treated with DMSO or MZ or vinorelbine or paclitaxel for 3 hours. They were then washed with PBS and then lysed with 100 µl of the lysis buffer (20 mM Tris-HCL, pH 6.8, 0.5% Nonidet P-40, 1 mM $MgCl_2$, 2 mM EGTA, 0.5 μg/ml paclitaxel). Following lysis, the cellular residues were scraped from the wells and transferred to a 1.5-ml tube. Each well was rinsed with a second 100 µl of the lysis buffer and combined with the first. After vigorous vortexing for 10 sec, the samples were centrifuged at 12,000 x g for 10 min at at 4°C. Supernatants containing soluble tubulin were separated from pellets containing polymerized tubulin and placed in separate tubes. The pellets were resuspended in 100 µl water. The cytosolic and cytoskeletal fractions were each mixed with 200 µl and 100 µl of 2 x SDS sample buffer (125mM Tris-HCL (pH 6.8), 4% SDS, 20% glycerol, 100mM DTT, 2x proteinase inhibitor cocktail (Complete, Roche,)), respectively. Following heating them at 95°C for 5 min, 20 µl of each sample was analyzed by immunoblotting using both monoclonal mouse anti-α-tubulin and anti-β-actin antibody (Sigma) as described above. The band of each sample was quantiated by NIH image, and the ratios of de-polymerized versus polymerized tubulin were calculated in each treatment sample. The ratio of polymerized versus polymerized actin were also determined as an internal control. The t-test was used to analyze the significant differences between the ratios of depolymerization/polymerization of tubulin or actin in control cells and those in treated cells. Significance was assumed for $p<0.05$.

7/21/02 J.S J.S Date 8/4/01

TITLE Immunostaining

Project No. 5
Book No. 2

From Page No.—

① To clearify BAX translocation from cytosol to mitochondaica
② To estimate microtable (spindle) formatim after MZ-tox

Cell : H460 and WI-38.

① Method. Using 2x chamber slides.

day 0 → day 1 → day 2
2×10^4/well Treatment Mito Tracker stain → Immunostaing.

<Mito Tracker staing>

1) 100nM in media is prepared using 1mM stock of Mito-red.
2) Replace culture media to Mito-containg media
3) Incubate for 30min.

↓

Immuno staing protocal (same as P55)

(BAX x 200)

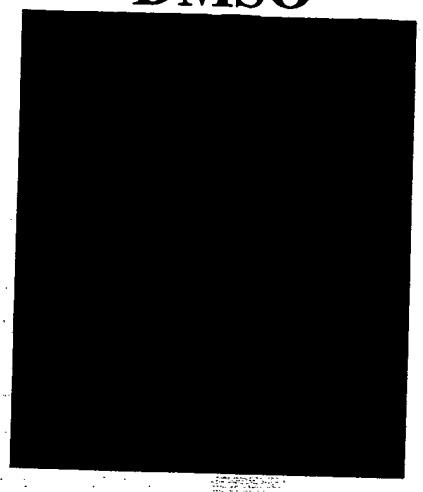
② Method. Same as P55

(α-Tubulin x 500)

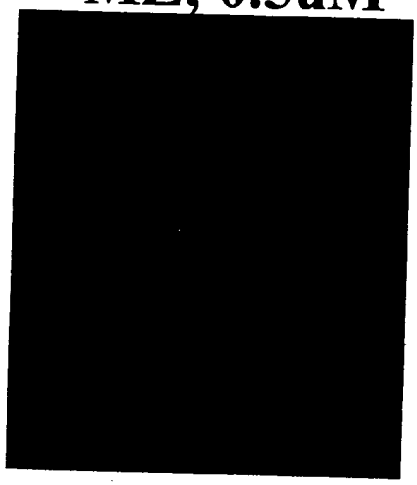
Inessed & Understood by me,	Date	Invented by	Date
Jozhi	2/21/02	J.S	8/5/01
		Recorded by J.S	

Tubulin staining in h460 CELL

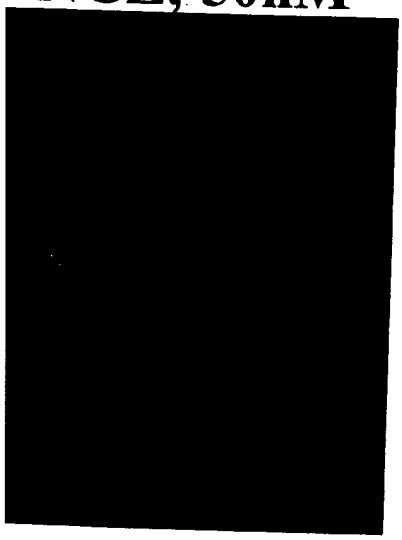
DMSO



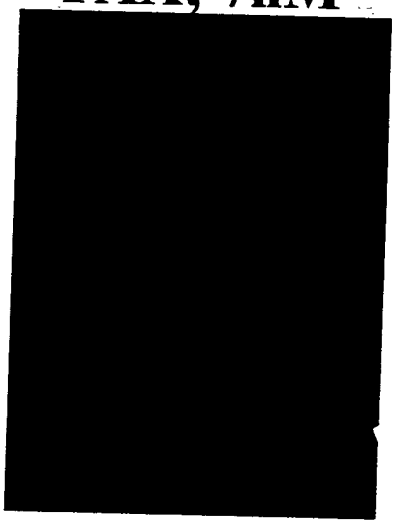
MZ; 0.5uM



NCZ; 50nM



TAX; 7nM



To Page No. 65

Yoshi

Date 2/21/02

Invented by J-S

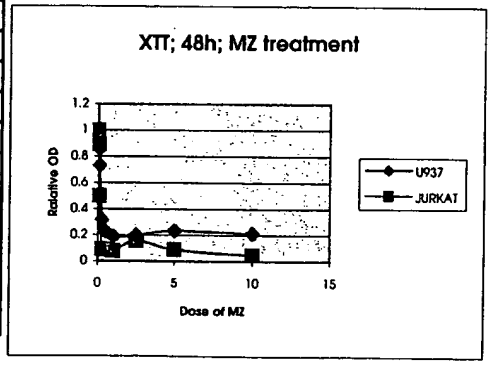
Recorded by J-S

Date 8/8/01

Design : Same as page 61
Cell : JURKAT, U937, MIYAPAKA-2

Row	data		8/7/01 U937		JURKAT			
	1	2	3	4	5	6	7	8
	0.13	1.128	1.142	1.067	1.031	0.862	0.449	0.363
	0.132	1.185	1.026	1.09	0.952	0.842	0.469	0.346
	0.133	1.073	1.11	1.059	1.003	0.792	0.453	0.379
	0.135	1.201	1.074	0.986	1.015	0.999	0.438	0.361
		0.816	0.783	0.75	0.801	0.477	0.21	0.19
		0.873	0.882	0.781	0.769	0.482	0.2	0.251
		0.792	0.838	0.875	0.724	0.491	0.209	0.18
	0.1325	0.947	0.807	0.86	0.81	0.522	0.175	0.166
	U937	0	0.01	0.025	0.05	0.1	0.25	0.5
		0.9955	1.0095	0.9345	0.8985	0.7295	0.3165	0.2305
		1.0525	0.8935	0.9575	0.8195	0.7095	0.3365	0.2135
		0.9405	0.9775	0.9265	0.8705	0.6595	0.3205	0.2465
		1.0685	0.9415	0.8535	0.8825	0.8665	0.3055	0.2285
	AVERAG	1.01425	0.9555	0.918	0.86775	0.74125	0.31975	0.22975
	RATIO	1	0.942	0.905	0.856	0.731	0.315	0.227
	JURKAT	0	0.01	0.025	0.05	0.1	0.25	0.5
		0.6835	0.6505	0.6175	0.6685	0.3445	0.0775	0.0575
		0.7405	0.7495	0.6485	0.6365	0.3495	0.0675	0.1185
		0.6595	0.7055	0.7425	0.5915	0.3585	0.0765	0.0475
		0.8145	0.6745	0.7275	0.6775	0.3895	0.0425	0.0335
	AVERAG	0.7245	0.695	0.684	0.6435	0.3605	0.066	0.06425
	RATIO	1	0.959	0.944	0.888	0.498	0.091	0.089

	U937	JURKAT
0	1	1
0.01	0.942	0.959
0.025	0.905	0.944
0.05	0.856	0.888
0.1	0.731	0.498
0.25	0.315	0.091
0.5	0.227	0.089
1	0.188	0.077
2.5	0.197	0.155
5	0.233	0.088
10	0.211	0.046



	JURKA
0	
0.01	
0.025	
0.05	
0.1	
0.25	
0.5	
1	
2.5	
5	
10	

Yoshi 2/21/02 J.S J.S Date 8/9/01

TITLE Tissue Hemoglobin Assay Project No.

Book No. 2

From Page No. According to user's manual.
For standard.

BECKMAN DU-600

Date: 08/31/01
Time: 01:41

Protein Analysis: Standards

Samples DispStdCurve ViewStats Method SaveClear Print Quit

Assay type: User defined
Standards file: A:\WORK_STD
Component name: ICHIRO
Curve fit: Linear, zero intercept
Sampling device: Auto smplr
Number of standards: 6
Read average time: 0.50 sec

Analytical wl: 600.0 nm
Method name: A:\HEM
Units: MG/DL

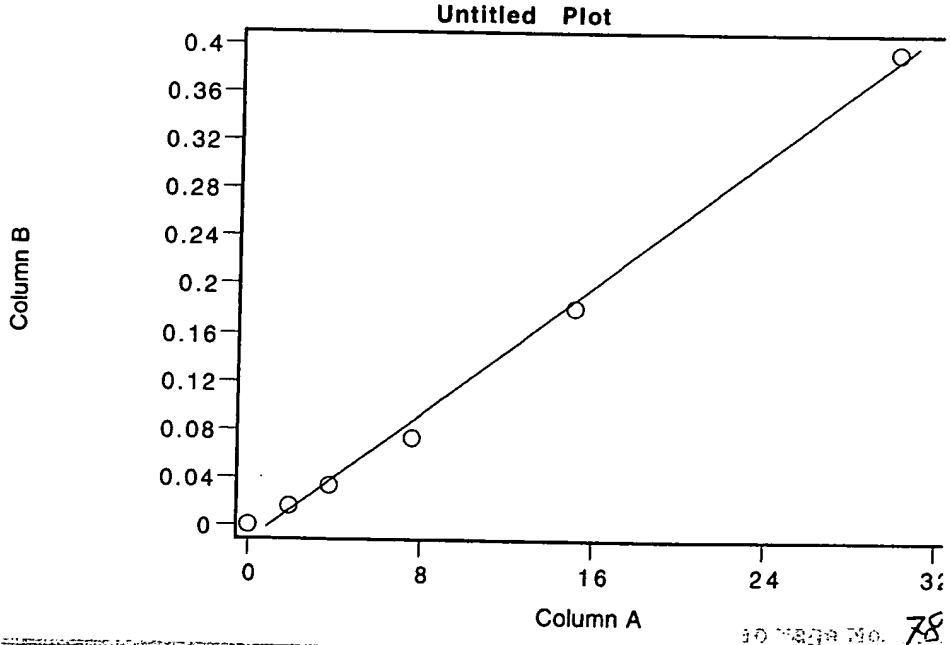
Number of replicates: 1
Flag standards over: 1.000% CV

Std#	Rep#	Std Conc	Calc Conc	Diff	%CV	Analytical abs	Use	Flag
Read 1		0.0000	0.1468	0.1468		0.0019	[Y]	
Read 2		1.8750	1.4012	-0.4736		0.0179	[Y]	
Read 3		3.7500	2.7566	-0.9934		0.0352	[Y]	
Read 4		7.5000	5.8401	-1.6599		0.0746	[Y]	
Read 5		15.000	14.2779	-0.7221		0.1823	[Y]	
Read 6		30.000	30.9298	0.9298		0.3948	[Y]	

Tissue Hemoglobin Assay /
Standard

mg/dl	0	1.875	3.75	7.5	15	30
Abs	0.0019	0.0179	0.0352	0.0746	0.1823	0.3948

Column A	Column B
0	0.0019
1.875	0.0179
3.75	0.0352
7.5	0.0746
15	0.1823
30	0.3948



Witnessed & Understood by me.

Date 2/21/02

Invented by J.S

Recorded by J.S

Date 8/31/01

Project No. ___

Book No. 2 TITLE Tissue Hemoglobin Assay

age No. 27 Sample : 5/6/00 Xenograft tumor.

Read average time: 0.30 sec

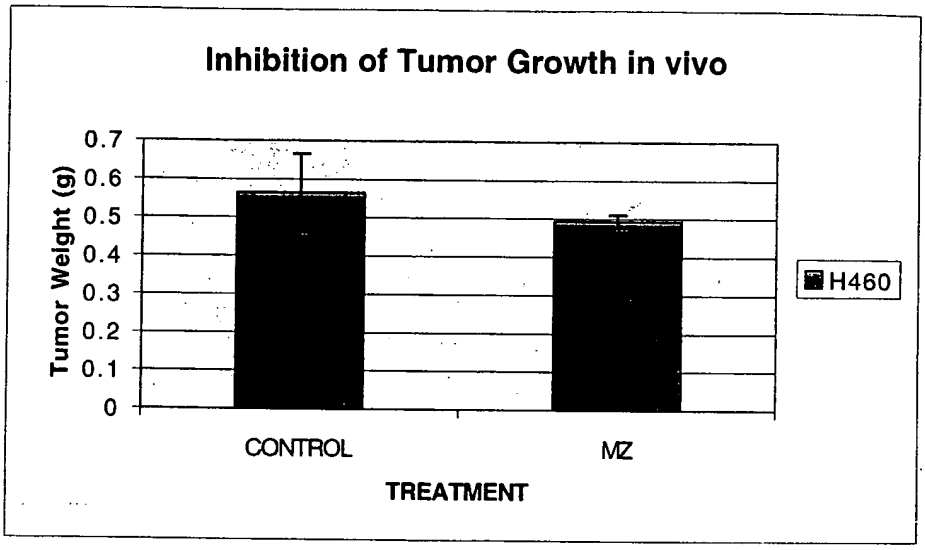
H460

Sample ID	Rep#	Analytical abs	5/6/00	Dilution Factor	Conc MG/DL	Flag Tumor volume
1		0.0094		1.0000	0.7350	0.42 g
2		0.0261	C	1.0000	2.0462	0.62 g
3		0.0223		1.0000	1.7479	0.65 g
4		0.0181		1.0000	1.4182	0.47 g
5		0.0236	M	1.0000	1.8451	0.51 g
6		0.0078	Z	1.0000	0.6077	0.5 g

TUMOR VOLUME

	CONTROL	MZ
1	0.42	0.47
2	0.62	0.51
3	0.65	0.5
ge	0.56333333	0.49333333
	0.10208929	0.01699673
		0.39297439

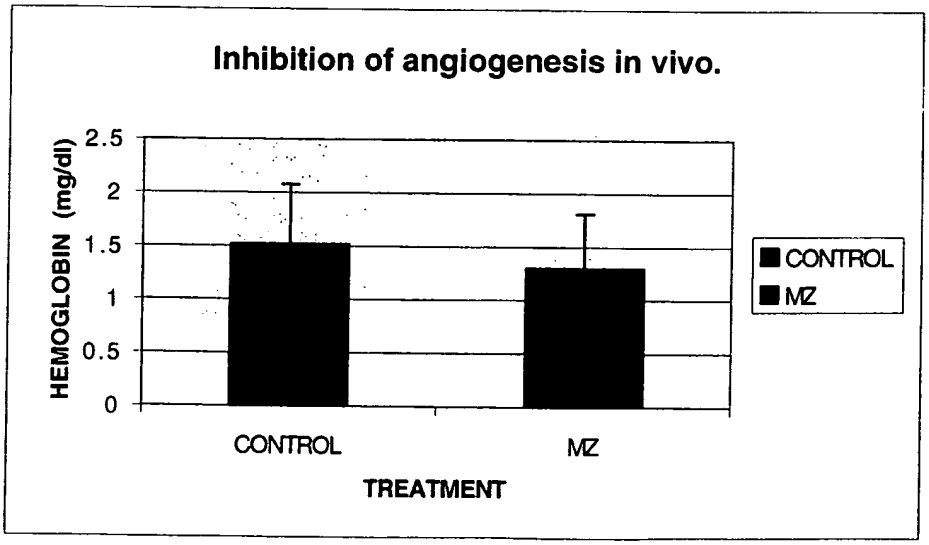
	CONTROL	MZ
	0.56333333	0.49333333



TISSUE HEMOGLOBIN

	CONTROL	MZ
1	0.735	1.4182
2	2.0462	1.8451
3	1.7479	0.6077
e	1.5097	1.29033333
	0.56116889	0.51319399
		0.70419558

	CONTROL	MZ
	1.5097	1.29033333



To Page No. ___

ed & Understood by me.	Date	Invented by	Date
Zhou	2/21/02	J-S	8/31/01
		Recorded by J-S	

TITLE Tissue Hemoglobin Assay

Project No. 3

Book No. 2

From Page No.

Re-experiment using same sample as page 78

Tissue Hemoglobin Assay 2

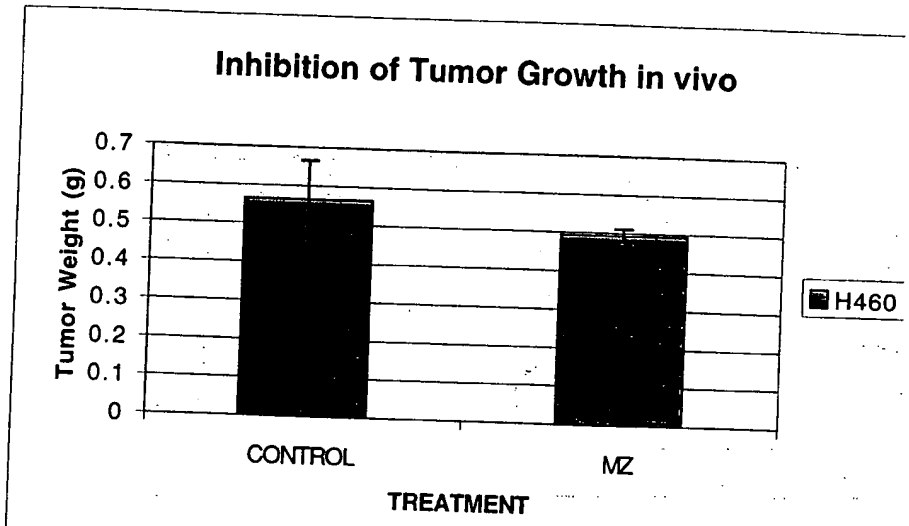
Standard

mg/dl	0	1.875	3.75	7.5	15	30
Abs	0.0004	0.0196	0.0372	0.0773	0.1508	0.3459

TUMOR VOLUME

H460	CONTROL	MZ
1	0.42	0.47
2	0.62	0.51
3	0.65	0.5
average	0.56333333	0.49333333
SE	0.10208929	0.01699673
p		0,39297439

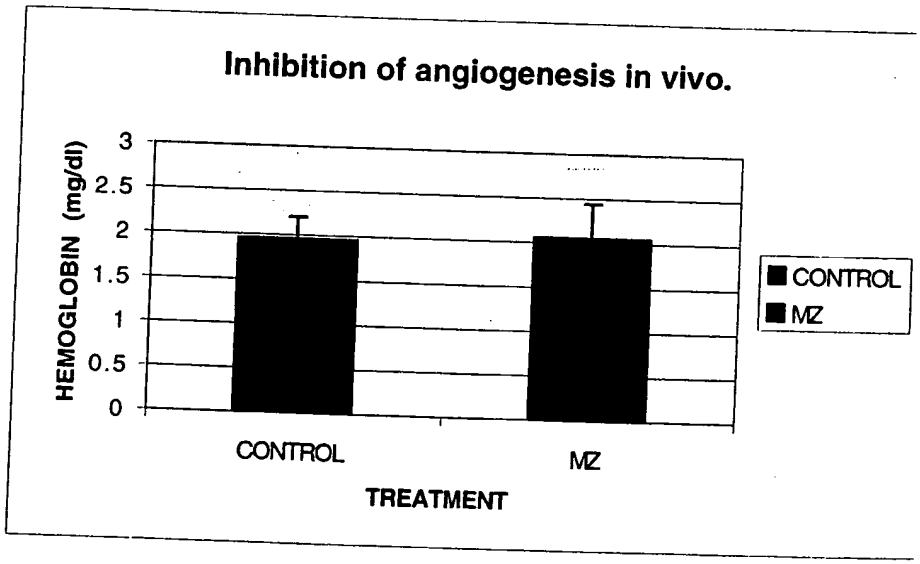
H460	CONTROL	MZ
	0.56333333	0.49333333



TISSUE HEMOGLOBIN

H460	CONTROL	MZ
1	1.75702	2.56919
2	2.30513	1.94098
3	1.80134	1.63065
average	1.95449667	2.04694
SE	0.24859454	0.39041429
p		0.79160352

H460	CONTROL	MZ
	1.95449667	2.04694



To Page No.

Witnessed & Understood by me: Zozhi 2/21/02

Date

Invented by J.S

Recorded by J.S

Date 9/7/01

EXHIBIT 3

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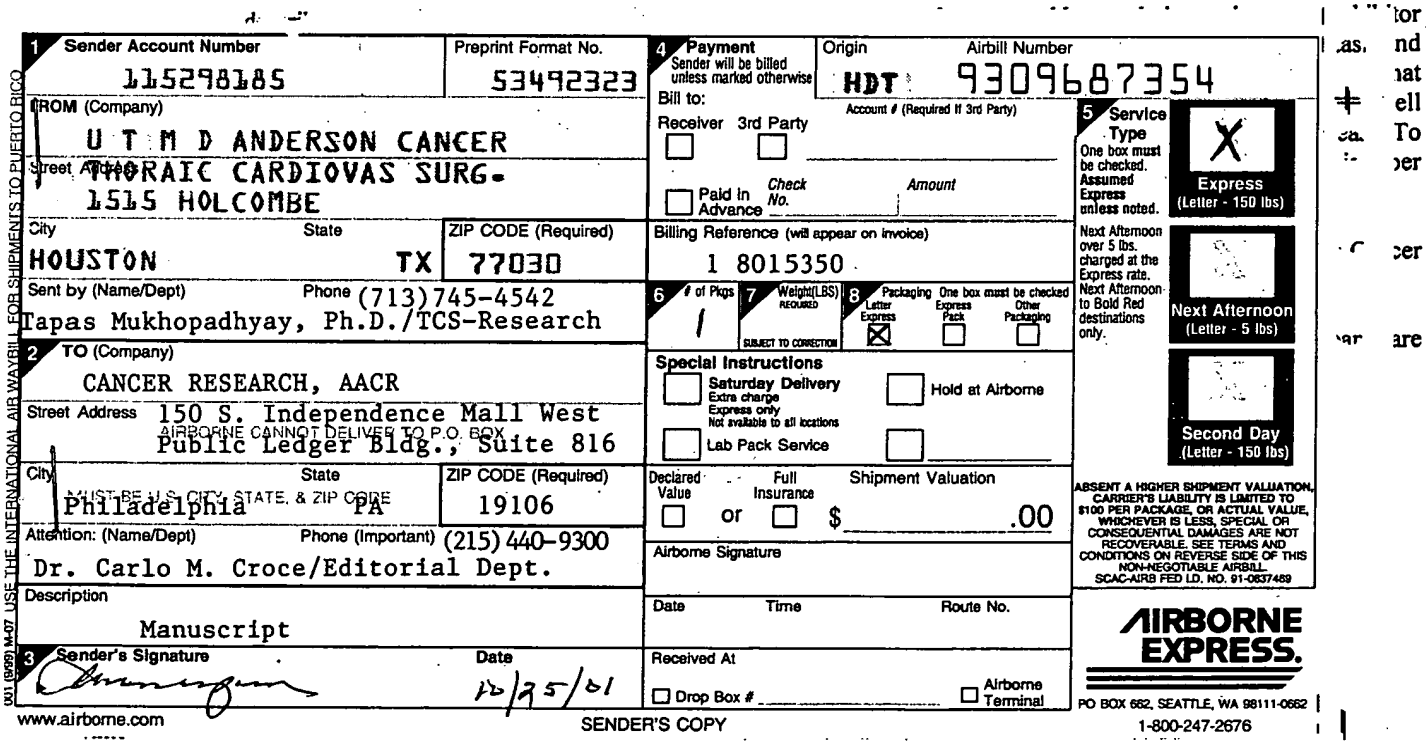
October 25, 2001

Dr. Carlo M. Croce
Editor-in-Chief
Cancer Research, AACR
150 S. Independence Mall West
Public Ledger Bldg., Suite 816
Philadelphia, PA 19106-3483

Dear Dr. Croce:

The manuscript entitled "**Mebendazole: A Novel Microtubule Agent Having Potent Antitumor Activity** "is being submitted for consideration for publication as an *Advances in Brief* article in ***Cancer Research.*** I am sending the cover letter in duplicate, four copies of the manuscript, and four sets of original illustrations. The subject category that applies to this manuscript is Experimental Therapeutics. Please charge in the amount of $75 for the submission fee in my credit card (VISA 4427-1000-0672-8518, Exp 04/02). I would like to suggest Dr. Jeffrey A. Norton as the Associate Editor.



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- Journal Name: Cancer Research Journal
- Deadline Date: _____
- Will you be generating your own cover letter? ☒ Yes ☐ No
- Is acknowledgement for grant support applicable? ☐ Yes ☐ No
  - EXAMPLE: "This study was partially supported by grants from the National Cancer Institutes and The National Institute of Health (P01 CA78778-01A1) [J.A.R.]; SPORE (2P50-CA70970-o4); by gifts to the Division of Surgery, from Tenneco and Exxon for the Core Laboratory Facility; by the UT M. D. Anderson Cancer Center Support Core Grant (CA 16672); by a grant from the Tobacco Settlement Funds as appropriated by the Texas State Legislature (Project 8), The W. M. Keck Foundation, and a Sponsored research agreement with Introgen Therapeutics (SR93-004-1)."
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Mebendazole: A Novel Microtubule Agent Having Potent Antitumor Activity[1]

Tapas Mukhopadhyay[2], Jichiro Sasaki, Rajagopal Ramesh, and Jack A. Roth

Department of Thoracic and Cardiovascular Surgery, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA.

Running Title: A novel microtubule agent as a potent anticancer drug.

Key Words: Tumor growth, angiogenesis, apoptosis, cell cycle, mebendazole

[1]Partially supported by grants from the National Cancer Institute, National Institutes of Health (P01 CA78778-01A1, to J. A. R.; a SPORE grant (2P50-CA70970-04); a developmental grant from the National Cancer Institute to The University of Texas M. D. Anderson Cancer Center SPORE grant for lung cancer (P50-CA70907; to T. M.); gifts to the Division of Surgery, at M. D. Anderson Cancer Center from Tenneco and Exxon for its Core Laboratory Facility; M. D. Anderson Support Core Grant (CA 16672); a grant from the Tobacco Settlement Funds as appropriated by the Texas State Legislature (Project 8); the W. M. Keck Foundation; and a sponsored research agreement with Introgen Therapeutics (SR93-004-1).

[2]Requests for reprints should be addressed at the Department of Thoracic and Cardiovascular Surgery, Box 445, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 745-4542; Fax: (713) 794-4901; E-mail: tmukhopa@mdanderson.org.

[2]The abbreviations used are: Mebendazole, mebendazole (5-benzoyl-2-benzimidazole carbamatic acid. The abbreviations used are: MZ, (methyl 5-benzoylbenzimidazole-2-carbamate; FZ, methyl 5-(phenylthio)-2-benzimidazole carbamate (fenbendazole; PBS, phosphate-buffered saline; NSCLC, non-small cell lung cancer; DMSO, dimethylsulfoxide; SDS, sodium dodecyl sulfate;

4/21/01 - COPY TO Dr. RAMESH FOR MT.

ABSTRACT

We have found that Mebendazole (MZ), a derivative of benzimidazole, induces a dose- and time-dependent apoptotic response in human lung cancer cell lines. In this study, MZ arrested cells at the G2/M phase before the onset of apoptosis as detected by using fluorescence-activated cell sorter analysis. MZ treatment also resulted in mitochondrial cytochrome-c release followed by apoptotic cell death. Additionally, MZ appeared to be a potent inhibitor of tumor-cell growth with little toxicity to normal WI38 and human umbilical vein endothelial cells. When administered orally to nu/nu mice, MZ strongly inhibited the growth of human tumor xenografts and significantly reduced the number and size of tumors in an experimental model of lung metastasis. In assessing angiogenesis, we found significantly reduced vessel densities in MZ-treated mice compared with those in control mice. These results suggest that MZ is effective in the treatment of cancer and other angiogenesis-dependent diseases.

INTRODUCTION

Microtubules serve as an intracellular scaffold, and their unique polymerization dynamics are critical for many cellular functions (1; 2). It is conceivable that cytoskeletal dysfunction, manifested as either a disrupted microtubule network or stabilized, "rigid" microtubule cytoskeleton, is an intracellular stress. Furthermore, disruption of the equilibrium between tubulin monomers/dimers and microtubule polymers using microtubule-stabilizing (paclitaxel, docetaxel) or -destabilizing (vinblastine, vincristine, nocodazole, colchicine) agents activates the stress-activated protein kinase signaling cascade. Such microtubule disruption is associated with G2/M-phase blockage (3-7). A number of microtubule drugs have been shown to be highly active, with significant clinical activity against tumor cells. However, the majority of these drugs are highly toxic which limits their application.

We have analyzed both the *in vitro* and *in vivo* effect of mebendazole (5-benzoyl-2-benzimidazole carbamatic acid; MZ), a derivative of benzimidazole (BZ), on tumor-cell growth as well as the molecular mechanisms involved in its action. BZ interacts weakly with host tubulin and affects the microtubule assembly only at high concentrations, while MZ is an anthelmintic drug that is used extensively for gastrointestinal parasitic infections in humans. However, the major application of these compounds to date, has been the treatment of veterinary and human helminthiasis, in which they have demonstrated remarkable efficacy and safety (8). In this study though, we were interested in determining the effect of MZ on solid tumor growth and angiogenesis. Structurally different from other anticancer drugs, MZ is remarkably safe at high doses in humans. We report here evidence indicating that MZ induces G2/M cell cycle arrest, which ultimately promotes apoptosis in lung cancer cells. Our results demonstrate for the first time the antitumor and antiangiogenetic effects of MZ both *in vitro* and *in vivo*.

MATERIALS AND METHODS

***In Vitro* Cell Culture and Proliferation Assay.** Cells of the human non-small cell lung cancer cell line A549 and WI38 normal fibroblasts (American Type Culture Collection, Rockville, MD) and H460 cells (a gift from Drs. Adi Gazdar and John Minna, The University of Texas Southwestern Medical Center at Dallas, Dallas, TX) were seeded on to culture plates (2 x 10^4 cells/well) in F12 and RPMI medium, respectively, supplemented with 10% heat-inactivated fetal calf serum and antibiotics. Also, human umbilical vein endothelial cells (HUVEC) were grown in medium supplemented with growth factor (Clonetics, San Diego, CA). When grown to 40% - 50% confluence, the cells were exposed to MZ dissolved in dimethyl sulfoxide (DMSO). Cell growth was monitored by counting the viable cells using a hemocytometer.

DNA Fragmentation and Cell Cycle Analysis. Control and MZ-treated cells were washed in cold phosphate-buffered saline (PBS). The cell pellets were lysed in lysis buffer (10 mM Tris, pH 7.4, 10,mM ethylenediaminetetra acetic acid, pH 8.0, 0.5% Triton X-100) and incubated for 10 min at 4°C and then incubated with 200 μg/ml RNase A for 1 h at 37°C. Following centrifugation, the supernatants were incubated with 200 μg/ml Proteinase K for 30 min at 50°C. Next, DNA fragments were precipitated with 0.5 M NaCl and 50% isopropanol and the samples were loaded in 2% agarose TBE gel and stained with ethidium bromide.

Growth of Tumor Xenografts in Nude Mice. Prior to the start of the experiments, mice underwent total-body irradiation (3.5 Gy). One day later, all animals received an injection of 2 x 10^6 H460 tumor cells into the lower right flank. Starting on the day after a 3- to 5-mm tumor was established, we administered an oral suspension of MZ at the indicated concentration every other day; five mice were used in each group. Both control and treated mice were then

monitored for tumor growth, with cross-sectional diameter of the tumors measured externally every 7 days. Also, the tumor volume was calculated as described previously (9). The experiments were conducted in triplicate.

Hemoglobin Assay. Quantitation of tumor vascularity was performed by using hemoglobin assay essentially as described previously (10). Briefly, subcutaneous tumors were excised, weighed, individually frozen in test tubes, and usually 24 h later, thawed. Approximately 20 ml of distilled water/gram of tumor tissue was then added, and the tumor was homogenized using a blade homogenizer until it was fully disintegrated. The debris was then pelleted via centrifugation (3000 x *g*, for 5 min), and the supernatant, which contained hemoglobin, was collected. The concentration of hemoglobin in the supernatant was determined according to the catalytic action of hemoglobin on the oxidation of 3,3',5,5'-tetramethylbenzidine by hydrogen peroxide as outlined by the manufacturer (Plasma Hemoglobin Kit; Sigma Chemical Co. St. Louis, MO)

Evaluation of Lung Metastases and Treatment *In Vivo*. To establish lung metastases, A549 tumor cells (10^6) were injected into the tail vein of 10 female nude mice as described previously (11). Six days later, we divided the mice into two groups of five each. Group 1 received no treatment, while group 2 received 1 mg of MZ orally (100 μl) twice a week for 3 weeks. After the 3 weeks, the animals were killed via CO_2 inhalation. The mice's lungs were then injected intratracheally with India ink and fixed in Fekete's solution. The therapeutic effect of MZ treatment was determined by counting (without knowledge of which was the treatment group) the metastatic tumors in each lung under a dissecting microscope. The experiments were performed three times, and data were analyzed and interpreted as being statistically significant if the P value was < 0.05 according to the Mann-Whitney test.

Histologic Analysis of Blood Vessels in Tumor Xenografts. Five-micrometer sections of paraffin-embedded tissue samples were stained with hematoxylin and eosin and subjected to immunoperoxidase detection of endothelial cells using a CD31 antibody (12). Vascular areas that stained positively for CD31 (at least five fields per specimen) were analyzed under bright-field microscopy. In all of the staining procedures, we included appropriate negative controls.

Chamber Assay of Angiogenesis. We used the dorsal air-sac method (13) to assay angiogenesis *in vivo*. Briefly, 1 x 10^7 cultured A549 cells were suspended in PBS and packed into round cellulose-ester membrane chambers having a diameter of 14 mm (pore size, 0.45 μm; Millipore Corporation, Bedford, MA). Each chamber was then implanted into a dorsal air sac of a nude mouse. From the next day onward, after implantation, the mice were given an oral suspension of MZ (1 mg/mouse/day); five mice were used in each group. The mice were killed on day 5, and the subcutaneous region overlying the chamber in each mouse was photographed.

Cellular Fractionation for Cytochrome-c and Western Blot Analysis. We performed cell fractionation using the Apo Alert Cell Fractionation Kit (Clontech, Palo Alto, CA) according to the user's manual. In brief, tumor cells were harvested and washed in washing buffer and then homogenized in lysis buffer in an ice-cold Dounce tissue grinder. The cell homogenates were then centrifuged at 700 x *g* for 10 min at 4°C. Afterwards, the supernatants were transferred into 1.5-ml tubes and centrifuged at 10,000 x *g* for 25 min at 4°C; they were then collected as cytosolic fractions, and the pellets were lysed in lysis buffer and collected as mitochondrial fractions. Western blot analysis was done as described earlier (14). Briefly, the protein concentration in both fractions was determined using the Bradford method (Bio-Rad, Hercules, CA). Next, 25 μg of protein was fractionated using sodium doderyl sulfate-polyacrylamide gel electrophoresis, transferred to Amersham

membranes (Amersham, Arlington Heights, IL), and immunoblotted with monoclonal antibodies against cytochrome-*c*, cytochrome-*c* oxidase subunit IV (COX IV), and β-actin. Immunoreactive proteins were detected using enhanced chemiluminescence (Amersham).

Statistical Analysis. To summarize the study results, we reported descriptive statistics, such as the mean and standard deviation. Also, two-sample *t*-test was performed to compare the tumors in control mice with those in mice treated under various conditions.

RESULTS

Effect of MZ on Tumor Cell Growth *In Vitro*. MZ treatment strongly inhibited the growth of the lung cancer cell lines (Fig. 1*A*); the half-maximal inhibitory concentration (IC_{50}) was ~0.16 μM. Specifically, MZ induced dose- and time-dependent inhibition of the growth of these cells. However, although MZ was highly cytotoxic to the tumor cells in culture, reducing their number to below the initial plating density, it had no effect on normal HUVEC or WI38 fibroblasts even at a concentration of 1 μM (Fig. 1*A*). Additionally, we examined the effect of MZ on H460 and A549 human lung cancer cells in a 5-day growth assay (Fig. 1*B*). We found that MZ inhibited growth of the cells fivefold compared with that of control cells. The growth-inhibitory effect was not restricted to lung cancer cells, as MZ also profoundly inhibited growth of breast, ovary, and colon carcinomas and osteosarcomas (Table 1), producing IC_{50} values that varied from 0.1 to 0.8 μM (data not shown). Table 1 also shows the growth-inhibitory effects of MZ on a number of tumor cell lines after 48 h of treatment.

Induction of G2/M Arrest Followed by Apoptosis by MZ in Lung Cancer Cell Lines. When cells were treated with varying doses of MZ, they w ere killed due to apoptosis. Specifically, H460 cells were exposed to MZ (0.2 to 5.0μM) for 24 h before the DNA was

extracted for agarose gel electrophoresis. Figure 1*C* shows that MZ induced DNA fragmentation at 24 h in a dose-dependent manner. The mechanism of cell death was determined to be apoptosis via detection of apoptotic cell populations that displayed a sub-2N genomic content during fluorescence-activated cells sorting (Fig. 1*D*). We found that the cells were blocked at the G2/M phase 12 h after MZ treatment before undergoing apoptosis. Cells were rounded and partly detached after 12 h of MZ treatment. However, after 24 h of treatment, cell shrinkage occurred, and nuclear bodies were evident; subsequently the cells underwent apoptosis. After 48 h of MZ treatment, more than 60% of the cells had undergone apoptosis with characteristic nuclear fragmentation (Fig. 1*D*). In addition, a number of apoptotic gene family proteins were examined using western blot analysis. It was found that p53 protein is post-translationally stabilized and elevated without an increase in mRNA (data not shown). This occurs so frequently during apoptosis induced by diverse stress stimuli that it has been considered a common feature of the apoptotic process. Furthermore, as a consequence of p53 stabilization, expression of the p53 target genes p21 and MDM2 was also induced. However, MZ had no effect on genes belonging to the Bcl2 family, including Bcl-xl, bax, bad, and bak, as determined via western blot analysis (data not shown).

Induction of Cytochrome-*c* Release and Caspase Activation by MZ. Because MZ could inhibit the growth of p53-null cell lines and other p53-mutated cells, although at a higher dose, we examined the other p53-independent pathways. To examine whether MZ signaling goes through a mitochondrial pathway, H1299 (p53-null) and H460 (wild-type) cells were treated with MZ in a dose-dependent manner, and cytosolic extracts lacking mitochondria were prepared and analyzed via immunoblotting (Fig. 1*E*). Cytochrome-*c* accumulated in cytosolic extracts at 12 h after exposure to MZ increased in both of the cell lines in a dose-dependent

manner. Also, the membranes were probed using an antibody against Cox IV, a protein that is specific for mitochondria, as an internal control. Both cell lines showed an increase in cytochrome-*c* protein in the cytoplasm after MZ treatment in a dose-dependent manner. Twenty-four hours after MZ treatment, activation of caspase-9 and caspase-8 and cleavage of the caspase substrate PARP and procaspase 3 were detectable (data not shown).

Inhibition of Tumor Cell Growth and Angiogenesis by MZ. The effect of MZ on the proliferation of tumor-cell lines *in vitro* prompted us to investigate its antitumor activity in a nu/nu mouse model. We established tumors in the mice by subcutaneously injecting them with 1×10^6 H460 cells, which are human non-small cell lung cancer cells. A dose-escalation study indicated that MZ suppressed growth of the tumors in a dose-dependent manner (Fig. 2*A*). Specifically, mice having established tumors (~3 mm in diameter) were fed 1 mg of MZ orally every other day, which was sufficient to profoundly inhibit tumor growth (Fig. 2*B*). The tumors were then harvested, photographed (Fig. 2*C*), and weighed. The experiment was repeated two times using 10 animals in both the control and treatment group. We found a marked difference in tumor weight between the MZ-treated and control animals (Fig. 2*D*). Additionally, in control mice, the xenograft of H460 cells exhibited a marked increase in tumor-growth kinetics compared with that in mice in the MZ-treated group. Furthermore, MZ-treated mice showed no signs of toxicity and were all healthier than the control mice were, during the 4 weeks of treatment (data not shown).

To determine whether the differences in growth kinetics observed *in vivo* were associated with variations in tumor vascularity, sections of subcutaneous tumors established from H460 cells were stained for CD31, a marker expressed by endothelial cells. A significant decrease in the number of CD31-positive endothelial cells was observed in MZ-treated mice when compared

with control mice. This analysis demonstrated substantially increased blood-vessel density in untreated mice compared with that in MZ-treated mice (Fig. 2*E*). Thus, MZ treatment profoundly reduced the neovascularization and growth of human lung cancer xenografts in nude mice. This tumor-suppressing effect of MZ may have been due to inhibition of tumor-induced angiogenesis. In addition, the tumor vascularity *in vivo* was quantitated in control and MZ-treated mice using a hemoglobin assay. The results of this assay indicated that there was a 75% reduction in hemoglobin content per gram of tumor sample obtained from MZ-treated mice, as compared with control mice (Fig. 2*F*).

*A*ngiogenesis *in vivo* was further assayed using the dorsal air-sac method (15) by photographing the area of subcutaneous neovascularization in mice overlying a semipermeable membrane chamber containing H460 or A549 cells. Twenty-four hours after each chamber was implanted, the animals were fed 1 mg of MZ orally every other day for a total of three treatments. Both the number and caliber of the blood vessels were significantly reduced in mice treated with MZ compared with those in control mice (Fig. 2*G*). To exclude the possibility that the reduced vasculature was due to a lack of viable tumor cells in the chamber, we prelabeled tumor cells using a fluorescent dye before injecting them into the chamber. After photographing the blood vessels, we examined the tumor cells on the membrane using a fluorescent microscope. The results indicated that the control and MZ-treated mice had similar cell densities on the membrane filters (Fig. 2*H*).

Next, we sought to determine whether MZ treatment would inhibit the growth of human lung cancer colony formation in an experimental lung metastasis model. In this study, about 300 metastatic colonies appeared in the lungs of control mice, 21 days after the injection of A549 cells via the tail vein (Figs. 3*A* and 3*B*). However, the oral administration of 1 mg of MZ per

mouse every other day reduced the mean colony count to 80% of the mean count in control mice ($P < 0.0001$). This experiment was performed three times with similar results. In another experiment, mice that were treated using paclitaxel alone did not show a significant reduction in colony formation (data not shown). Histochemical staining of lung tissues using hematoxylin and eosin indicated that not only the number but also the size of the metastatic tumor colonies (as measured according to the transverse diameter of the tumor colony) was substantially reduced by treatment using MZ (Fig. 3*C*).

Discussion

MZ is one of the truly broad-spectrum anthelmintics, the BZs, which have a high therapeutic index. Central to the success of the BZs is their selective toxicity in helminths. Although the diverse activities of these compounds have been described at both the biochemical and cellular level, their molecular mechanism of action has not been explored in detail; when it has been studied, this mechanism has proven to be controversial. BZs are known to inhibit a wide variety of apparently unrelated mechanisms. Of these mechanisms, fumarate reductase, glucose uptake, and microtubule inhibition satisfy many of the criteria considered relevant for a putative site of action. This gives rise to the question of whether these mechanisms are directly or indirectly related. Based on the inhibitor profile of both fumarate reductase and glucose uptake, it is apparent that these systems are not specific to BZs. The coincidence of three structurally distinct microtubule-inhibitor classes acting on these mechanisms supports the hypothesis of microtubule dependence; however, this is complicated by the activity of noninhibitors in both techniques.

There are sufficient data supporting a general concept of primary microtubule action leading to a series of biochemical effects that either directly or indirectly elicit a number of

changes; as we demonstrate here, these changes vary in normal and cancer cells. The results of binding studies using enriched extracts from the tubulin of helminths and mammals have suggested that tubulin are the substrate of BZs (8; 16; 17). However, the results of crystallographic and other studies have indicated that the tubulin-binding site of BZs is distinctly different from that of other microtubule-disrupting agents like vinblastine and paclitaxel (taxol). Drugs in the latter group bind to tubulin at sites located near the intradimer interface and facing the lumen of the microtubule, whereas the possible binding site for BZs is on the outside of the microtubule (18; 19). Although BZs are potent inhibitors of tubulin, a comparison of their relative activity showed that some structural refinement produces BZ derivatives, like MZ, that are significantly less active against mammalian tubulin, although their broad structural specificity remains unchanged. Also, some BZs have been reported to have poor systemic absorption after oral administration *in vivo*; the exception is MZ, which has been shown to have an absorption profile of >50% (20). The observed safety of the BZs as anthelmintics may also be unrelated to BZ-tubulin binding but rather may be due to differences in the metabolic or detoxification pathways as suggested by (21). For example, rapid, extensive metabolism of BZs into less-toxic metabolites (*e.g*, sulfoxides and sulfones) by the hepatic microsomal enzymes (22; 23) may account for some of the lack of host toxicity. Parasites, on the other hand, lack these metabolic pathways and are killed by BZs. Additionally, some terbenzimidazole compounds have been reported to be topoisomerase I poisons (24); therefore, we tested MZ for such an effect but could not detect it (unpublished results).

The effect of MZ as an antitumor agent has never been tested before. With that in mind, one of our most encouraging findings was that MZ inhibited neovascularization both *in vitro* and in the human xenografts we tested, indicating that MZ is a potent antiangiogenic agent.

Moreover, it had no effect on normal endothelial cell growth but directly targeted tumor cells *in vivo*. Although the molecular mechanism of MZ action on tumor-growth inhibition requires further elucidation, our results show that MZ may be effective in the treatment of cancer and other angiogenesis-dependent diseases.

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REFERENCES

1. McNally,F.J. Modulation of microtubule dynamics during the cell cycle. Curr Opin.Cell Biol, *8*: 23-29, 1996.

2. Saunders,C. and Limbird,L.E. Disruption of microtubules reveals two independent apical targeting mechanisms for G-protein-coupled receptors in polarized renal epithelial cells. Journal of Biological.Chemistry., *272*: 19035-19045, 1997.

3. Manfredi,J.J. and Horwitz,S.B. Taxol: an antimitotic agent with a new mechanism of action. Pharmacol Ther, *25*: 83-125, 1984.

4. Rowinsky,E.K. and Donehower,R.C. Paclitaxel (taxol). N Engl J Med, *332*: 1004-1014, 1995.

5. Bhalla,K., Ibrado,A.M., Tourkina,E., Tang,C., Mahoney,M.E., and Huang,Y. Taxol induces internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia cells. Leukemia, *7*: 563-568, 1993.

6. Long,B.H. and Fairchild,C.R. Paclitaxel inhibits progression of mitotic cells to G1 phase by interference with spindle formation without affecting other microtubule functions during anaphase and telephase. Cancer Res, *54*: 4355-4361, 1994.

7. Horwitz,S.B. Mechanism of action of taxol. Trends Pharmacol.Sci., *13*: 134-136, 1992.

8. Lacey,E. The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. Int.J.Parasitol., *18*: 885-936, 1988.

9. Huober,J.B., Nakamura,S., Meyn,R., Roth,J.A., and Mukhopadhyay,T. Oral administration of an estrogen metabolite induced potentiation of radiation antitumor effects in presence of wild-type p53 in non-small cell lung cancer. Intl J Rad Oncol Biol Phys, *48*: 1127-1137, 2000.

10. Parish,C.R., Freeman,C., Brown,K.J., Francis,D.J., and Cowden,W.B. Identification of sulfated oligosaccharide-based inhibitors of tumor growth and metastasis using novel in vitro assays for angiogenesis and heparanase activity. Cancer Res, *59*: 3433-3441, 1999.

11. Kataoka,M., Schumacher,G., Cristiano,R.J., Atkinson,E.N., Roth,J.A., and Mukhopadhyay,T. An agent that increases tumor suppressor transgene product coupled with systemic transgene delivery inhibits growth of metastatic lung cancer *in vivo*. Cancer Res., *58*: 4761-4765, 1998.

12. Schumacher,G., Kataoka,M., Roth,J.A., and Mukhopadhyay,T. Potent antitumor activity of 2-Methoxyestradiol in human pancreatic cancer cell lines. Clin.Cancer Res., *5*: 493-499, 1999.

13. Bouvet,M., Ellis,L.M., Nishizaki,M., Fujiwara,T., Liu,W., Bucana,C.D., Fang,B., Lee,J.J., and Roth,J.A. Adenovirus-mediated wild-type p53 gene transfer downregulates vascular endothelial growth factor expression and inhibits angiogenesis in human colon cancer. Cancer Res., *58*: 2288-2292, 1998.

14. Nakamura,S., Roth,J.A., and Mukhopadhyay,T. Multiple lysine mutations in the c-terminal domain of p53 interfere with MDM2-dependent protein degradation and ubiquitination. Mol.Cell.Biol., *20*: 9391-9398, 2000.

15. Tanaka,N.G., Sakamoto,N., Inoue,K., Korenaga,H., Kadoya,S., Ogawa,H., and Osada,Y. Antitumor effects of an antiangiogenic polysaccharide from an arthrobacter species with or without a steroid. Cancer Res., *49*: 6727-6730, 1989.

16. Lubega,G.W. and Prichard,R.K. Specific interaction of benzimidazole anthelmintics with tubulin from developing stages of thiabendazole-susceptible and -resistant Haemonchus contortus. Biochem Pharmacol, *41*: 93-101, 1991.

17. Lacey,E. and Prichard,R.K. Interactions of benzimidazoles (BZ) with tubulin from BZ-sensitive and BZ-resistant isolates of Haemonchus contortus. Mol.Biochem.Parasitol., *19*: 171-181, 1986.

18. Hamel,E. Antimitotic natural products and their interactions with tubulin. Med Res Rev, *16*: 207-231, 1996.

19. Downing,K.H. Structural basis for the interaction of tubulin with proteins and drugs that affect microtubule dynamics. Annu Rev Cell Dev Biol, *16*: 89-111, 2000.

20. Van den,B.H., Rochette,F., and Horig,C. Mebendazole and related anthelmintics. Adv Pharmacol Chemother, *19*: 67-128, 1982.

21. Nare,B., Lubega,G., Prichard,R.K., and Georges,E. p-Azidosalicyl-5-amino-6-phenoxybenzimidazole photolabels the N- terminal 63-103 amino acids of Haemonchus contortus beta-tubulin 1. Journal of Biological.Chemistry., *271*: 8575-8581, 1996.

22. Lanusse,C.E., Nare,B., Gascon,L.H., and Prichard,R.K. Metabolism of albendazole and albendazole sulphoxide by ruminal and intestinal fluids of sheep and cattle. Xenobiotica, *22*: 419-426, 1992.

23. Lanusse,C.E., Nare,B., and Prichard,R.K. Comparative sulphoxidation of albendazole by sheep and cattle liver microsomes and the inhibitory effect of methimazole. Xenobiotica, *23*: 285-295, 1993.

24. Pilch,D.S., Xu,Z., Sun,Q., Lavoie,E.J., Liu,L.F., Geacintov,N.E., and Breslauer,K.J. Characterizing the DNA binding modes of a topoisomerase I-poisoning terbenzimidazole: evidence for both intercalative and minor groove binding properties. Drug Des Discov., *13*: 115-133, 1996.

Table 1. *Growth-inhibitory effect of MZ on other human tumor cell lines*[a]

Cell line	Tumor origin	Cell survival (%)
MCF-7	Breast	24.26 ± 8.4
H322	Lung	68.50 ± 3.7
H226Br	Lung	64.24 ± 0.2
H358	Lung	73.45 ± 1.8
Saos-2	Osteosarcoma	65.36 ± 10.1
SW480	Colon	63.63 ± 13.3
MDA 231	Breast	66.24 ± 15.8
SK-OV-433	Ovary	28.15 ± 6.1
RD	Rhabdomyosarcoma	72.24 ± 2.8
HT1080	Osteosarcoma	44.49 ± 7.7

[a]The viability of the cells was measured using a trypan blue exclusion assay after 48 h of MZ treatment (0.16 μM). The 100% value was derived from measurements obtained from untreated cells. Experiments were done in triplicate.

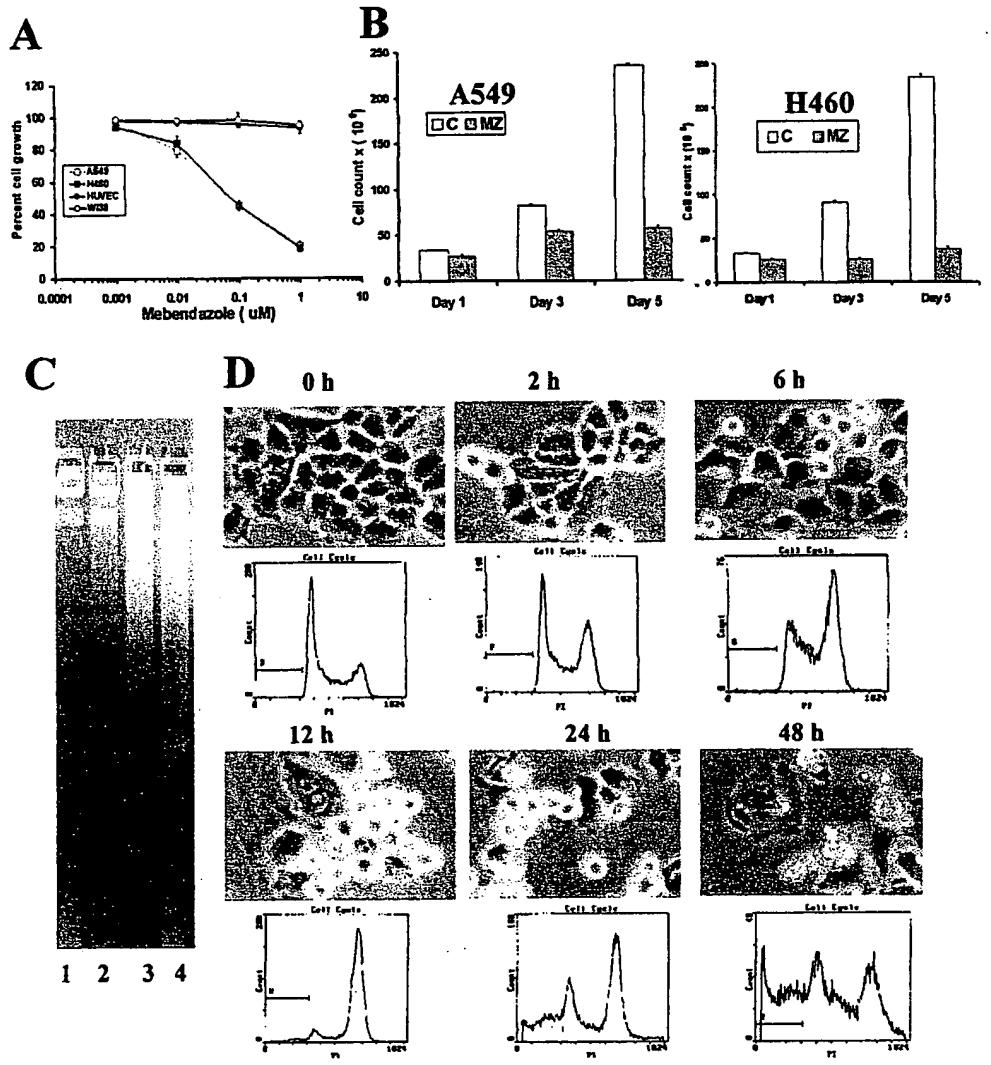
FIGURE LEGENDS

Fig. 1. Effect of mebendazole (MZ) on cell growth and apoptosis. ***(A)***, Dose-dependent inhibition of cell proliferation after MZ treatment. The H460, A549, HUVEC, and WI38 cell lines were used in this assay. **(B),** H460 and A549 cells were treated with 0.165 μM MZ (IC_{50}), and a 5-day growth assay was done. ***(C)***, dose-dependent DNA fragmentation analysis was done in H460 cells after 24 h of MZ treatment. Lane 1, controls; lanes 2-4, H460 cells exposed to 0.2, 0.5, and 1.0 μM MZ. ***(D)***, H460 cells treated with 0.2 μM MZ, harvested at different time intervals, and stained with PI. The cells were processed for fluorescence-activated cell sorter analysis to determine the cell cycle phase and apoptosis. Subdeploid populations indicated the apoptotic cells. The phase-contrast photomicrographs show mitotic cells after 12 h of MZ treatment and apoptotic nuclei after 24 and 48 h of MZ treatment. ***(E)***, Cytochrome-*c* detected using western blot analysis in the cytosolic fraction of H1299 and H460 cells. A considerable increase in cytochrome-*c* was noticed, which correlated with the MZ dose. An antibody against COX IV, a mitchondria specific protein, was used to probe the membrane to eliminate the possibility of contamination during fractionation.

Fig. 2. Effect of MZ on tumor growth and angiogenesis. ***(A)***, MZ inhibited H460 xenograft tumor growth in athymic nu/nu mice in a dose-dependent manner. H460 cells were injected into mice (2×10^6 cells/mouse), and mice having established tumors (3-4 mm in diameter) were fed different concentrations of MZ (T02, T04, and T08 represent 200, 400, and 800 μg of MZ, respectively) every other day while control animals received

PBS. (***B***), Significant growth inhibition was observed when mice were fed 1 MS of MZ every other day. Open circle control mice; closed circle, MZ-treated mice. (***C***), Tumors were excised from control and MZ-treated mice after 4 weeks and photographed. A few mice were killed at the start of treatment when the tumor had reached 3-4 mm in diameter (***D***), Graphic representation of the weight (mg $\pm$ standard deviation) of tumors in control and MZ-treated mice on day 28. (***E***), Histologic analysis of blood vessels in H460 xenograft tumors via immunoperoxidase detection of endothelial cells using a CD31 antibody. Con. control mouse; MZ-treated mouse. (***F***), Plot of milligrams of hemoglobin per gram of tumor tissue obtained from control and treated animals following hemoglobin assay. (***G***), Effect of MZ on angiogenesis *in vivo*. Chamber assay showing that MZ inhibited capillary formation using A549 cells. It should be noted that the control implant had a tree-like architecture of major vessels (arrows) connecting to minor branches (arrowheads) but that the MZ-treated implants had scarce vessels. Con, control; MZ, treated. (***H***), A549 cells prelabeled using a fluorescent cell marker were detected on the chamber membranes of control (Con) and MZ-treated (MZ) mice.

Fig. 3. Effect of MZ treatment on lung colony formation in an experimental metastasis model. (***A***), A549 cells formed colonies on lung surfaces when injected through the tail vein. C, control mouse; T, MZ-treated mouse. The white spots on the lung surfaces are colonies (arrows). **(B)**, Quantitation of lung colonies in control and MZ-treated animals ($P < 0.0001$). (***C***), Hematoxylin and eosin-stained lung sections showing the sizes of tumor colonies in control (Con) and MZ-treated (MZ) animals. The colonies are indicated by dotted lines.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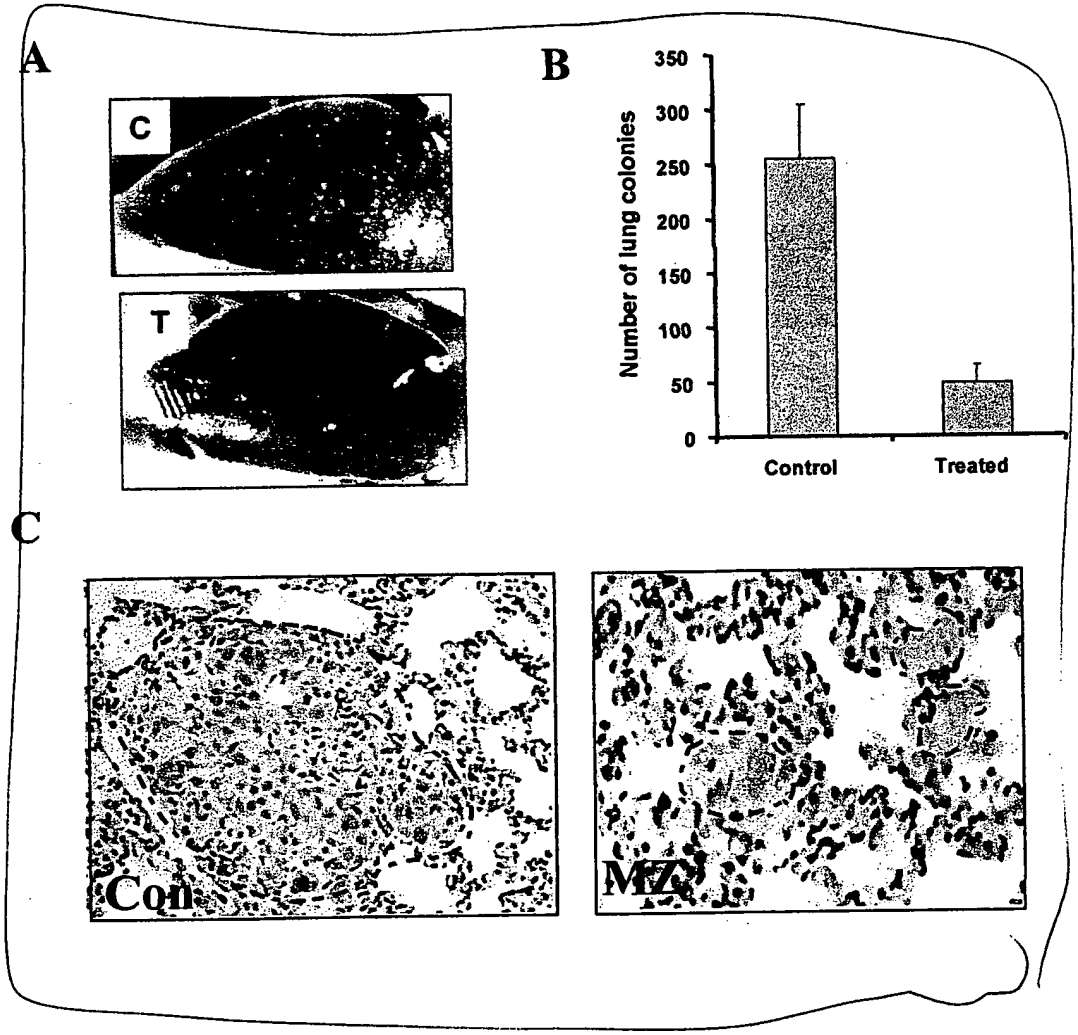
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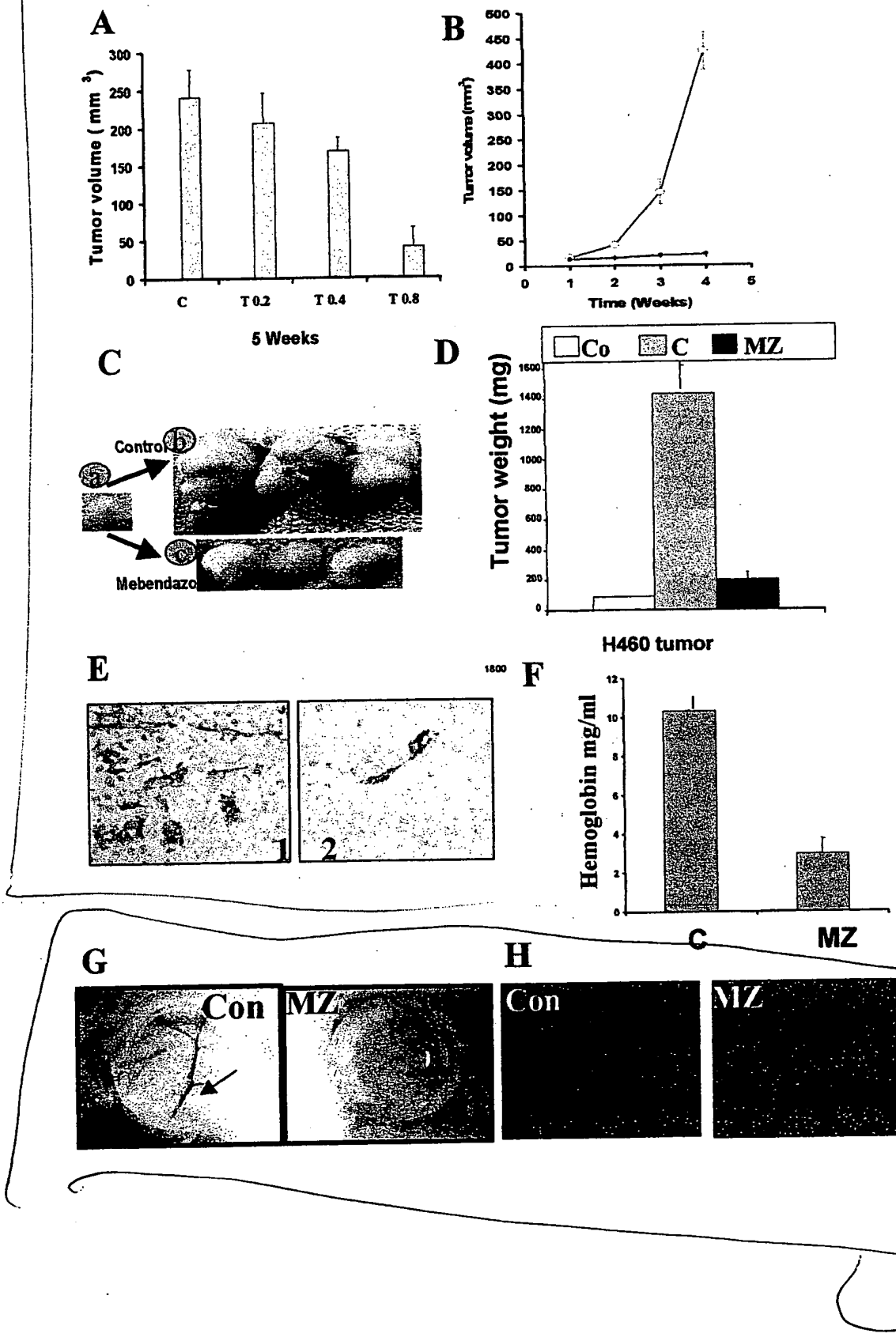
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